

One way out of a patent quagmire

The European Patent Office is caught in a taxonomic trap unintentionally set by its constitution. The only sensible way out that favours supporters of biotechnology comes with its own set of problems.

LAWYERS are fond of fuzziness, for obvious reasons. So they must adore the European Patent Convention, which has its fair share of legal ambiguities, is outdated, and is obstructing the development of industrial plant and animal biotechnology in Europe in a debate that rests on one of science's great but fuzzy disciplines: taxonomy. Ultimately the convention will have to be rewritten, but in a way that is bound either to undermine the biotechnology industry or enrage those who oppose it on ethical grounds.

The convention forms the constitution of the European Patent Office (EPO) — a body that currently finds itself at an impasse that prevents it from patenting genetically modified plants or animals, to the great frustration of its senior officials and of a burgeoning industry that they seek to serve. The convention was written in 1973, just before the advent of recombinant DNA technology, though it is only now that its shortcomings are coming home to roost.

Every one of the eight patents on higher organisms issued by the EPO since 1989 has been attacked on ethical grounds. Opponents have usually invoked the article in the convention which states that patents should not be issued if they are contrary to '*l'ordre publique*'. This 'morality clause' was inserted to exclude, for example, pornography and sophisticated weaponry from patentability, and anti-biotechnologists have never deployed it successfully.

The tide turned in February last year, during an appeal against a patent granted to the Belgian company Plant Genetics Systems (PGS) covering a procedure for producing herbicide-resistant plants, as well as the plants and seeds arising from the process. The 'opposition board', which is the first port of call for such appeals, insisted that the scope of the patent be restricted to exclude the plants produced, not on morality grounds, but rather on the basis of another exclusion clause in the convention, intended originally to protect plant breeders' rights. The clause states that modified animals and plants are patentable, but that new animal and plant varieties are not. That is where taxonomy's fuzziness has added to the lawyers' fun. Its first contribution was to give opponents the wherewithal to persuade the opposition board that plants can be considered collections of plant varieties and are therefore not patentable. Hence the impasse.

With hundreds of plant and animal applications pending, the EPO has sought ways of overturning that ruling on the basis of conflicting precedents, but, following the collapse of an opportunity last week (see page 178), so far in vain. It could, without significant further ado, change its implementation rules, instructing patent examiners and its legal staff to note that, for the purposes of patenting, a modified species is not to be considered as a collection of varieties for the purposes on patenting. Because such a unilateral judgement would cause an outcry, the EPO might sensibly wish to await the passing by the European Parliament and ministers of the European Commission's new directive on biotechnology which, in its current form, explicitly backs such a move. The EPO is not a European Union body, but an EU directive would give the office the political weight it requires to dare to change its implementing rules, if the directive is passed as it stands.

That is a big "if". The approach flies in the face of some countries' reluctance to support genetic technologies, and is also contrary to scientific sense. The definitions of variety and of species

have indistinct boundaries. Introducing genetic modifications adds yet another source of ambiguity to the debate — and one that can span taxonomic categories. Attempts to forcibly restrict the legal definition of 'variety' would surely fail — no form of words seems able to bypass that fundamental fuzziness.

Unfortunately for the EPO, the only sustainable way out of this impasse will encounter lengthy delay, controversy, and hostile voters: it must persuade its member states to rewrite the convention to remove the obstacle to patenting varieties. Such a step has a precedent: a few years ago the convention was changed to extend the proprietary period of pharmaceuticals. The current issue is much more controversial — years of work on the convention could end in political failure. But, if the law is to make sense, the EPO has no other choice.

In the meantime, the EPO can only hope that some expedient form of words can after all be found that will temporarily fill the gap, while its ethically motivated opponents should give thanks for taxonomy's limitations. □

Defend studies of science

Stanford should be developing programmes that bring together science and the humanities, not closing them down

SCIENCE, Technology and Society (STS) is a discipline taught and studied in many different forms in the United States, but always tries to engage experts in politics and economics, as well as sociology, in an exchange of ideas with scientists and engineers. Regrettably, the fact that it was spawned in the 1960s and early 1970s, and that some of its practitioners have embraced extreme positions (characterizing science as a social construct, for example), has fuelled a backlash against it. Some scientists have decided that, in these straitened times, criticism from social scientists is a luxury that they can do without.

Senior faculty at Stanford University in California don't think that: as their university is arguably the intellectual home of the silicon technology which is now transforming our society at an unprecedented rate, it would be a shame if they did. But the university is considering the abandonment of courses in STS (see page 183) because its interdisciplinary status makes it unrewarding for faculty members to spend time on it, rather than on more pressing demands inside their own departments. That is a problem for all interdisciplinary projects: however good they are, they can be the first to suffer from financial retrenchment.

Last year, some staff at Stanford put forward an ambitious proposal to expand STS into graduate teaching and research, thus providing the intellectual impetus to get faculty involved, raise standards and secure the programme's future. The engineering school says it can't afford it. The university must now step in, save the programme and establish a structure that will rescue it from the game of pass-the-parcel that has afflicted it since its 1971 inception.

The chasm between science and the humanities is still huge. If Stanford — excellent in both — won't keep trying to bridge it, who will? □



Student 'targets' may give boost to strategic planning in South Africa

Cape Town. Far-reaching changes to South African universities have been proposed by a national commission set up by President Nelson Mandela last year to advise, among other things, on revising the financing of tertiary institutions to keep the growth in student numbers in line with manpower requirements.

In particular, the National Commission for Higher Education, chaired by Jairam Reddy, a former rector of the University of Durban-Westville, has suggested that the research and teaching components of a university's support from the government be funded separately, with limits on the number of students in different disciplines — a move that might encourage more black students to take up science and engineering.

The commission, whose recommendations are expected to form the basis for future higher education policy, does not make any specific proposals about criteria for research funding, apart from saying that funds allocated for research be earmarked for that purpose.

But it does suggest that teaching funds should be allocated on the basis of fixed subsidies for each place on a degree course, based on the costs of training a student in the discipline concerned. The state would decide the overall number of subsidized places in each discipline, and individual institutions would negotiate their share of places through a new Higher Education Council.

Such a system would contrast sharply with the present funding system, in operation since 1982, under which university funding is based on a formula combining student enrolment and success rates, but with different weighting for arts- and science-based courses, and undergraduate and postgraduate students, as well as research output.

With no limitations on student numbers in different disciplines, this system has led to uncoordinated growth in South Africa's universities, concentrated in the arts and social sciences. This is mainly because growth has reflected increased numbers of black students, most of whom cannot register for science, medicine or engineering courses because they have not been taught mathematics and science to school-leaving level.

Furthermore, the state has never fully implemented the subsidy formula. At present, for example, it provides only two-thirds of the entitlement of universities and technikons (see *Nature* 379, 666; 1996), which must make up the balance through fees.

The commission expects further growth in the numbers in higher education, and points out that overall participation rates for the 20–24-year-old age group in South Africa are low compared to those in industrialized countries. This is because, while participation rates for whites (53 per cent) and Indians (61 per cent) are higher than in any country other than the United States, that for black Africans is only 12 per cent.

Universities would still be able to levy fees under the proposed system. But the fees would have to be approved by the Higher

should receive earmarked funds to extend their own student financial aid programmes. Earmarked funds are also proposed for staff development, building schemes and academic support for disadvantaged students.

These measures are aimed specifically at providing assistance to historically black institutions. Although all institutions in South Africa are now non-racial, universities with a higher proportion of black students tend to charge lower fees than their historically white counterparts, and on average receive less income per registered student on the basis of the current subsidy formula, on account of lower success rates and lower research output.

All those likely to be affected by such changes have until the end of May to comment on the discussion document, before the commission compiles its final report. Reaction so far has been mixed.

In particular, those attending a conference of 'stakeholders' earlier this month reached a general consensus that the issue of financial aid had not been adequately addressed. If tertiary institutions are to continue to levy fees, some comprehensive system has to be devised for providing assistance to students who are ineligible for loans from commercial banks because their parents are too poor to stand surety.

Some student bodies have been strongly critical. On 10 May, for example, student members of the South African National Students' Congress marched to the Johannesburg offices of the Education Ministry in the province of Gauteng to protest that the commission had "failed to address the issue of transformation in tertiary education institutions". The protesters also complained that students had not been represented on the commission.

Michael Cherry

Facing changes: students picket Gauteng's Education Ministry in Johannesburg.

Education Council. The National Student Financial Aid Scheme, launched earlier this year to provide loans to students for both fees and maintenance, would be extended.

The commission recommends the establishment of a national clearing house for applications. But it is not clear whether this would be an independent agency, a collective effort by the tertiary sector, or the responsibility of the Department of Education, which at present lacks the infrastructure to operate this kind of system.

A further proposal is that institutions

Republicans seek freeze on health research

Washington. Two Republican committees in the US Congress approved 'blueprints' for the 1997 federal budget last week that assume a freeze in spending on the National Institutes of Health (NIH) at its 1996 level of \$11.94 billion.

The blueprints are part of a seven-year strategy aimed at eliminating deficit spending, \$144 billion this year, by the year 2002. The plan would hold the NIH spending level constant over that period. In contrast, President Bill Clinton's 1997 budget request, submitted to Congress in March, had asked for an increase in the NIH budget of 3.9 per cent, to \$12.4 billion.

The two 1997 budget resolutions, passed by the House and Senate Budget Committees respectively, now go to appropriating committees, which will use them as maximum limits in drafting spending bills, although they can change particular allocations within their jurisdictions.

John Porter (Republican, Illinois), for example, chair of the House of Representatives subcommittee responsible for NIH funding, has vowed to raise NIH spending for the 1997 fiscal year. In particular, he wants to see an increase of 6 or 6.5 per cent for extramural scientists.

Meredith Wadman

Withdrawal of patent claim leaves position of plants unclarified

Munich. The European Patent Office (EPO) was last week deprived of the opportunity to reassess its current *de facto* moratorium on the patenting of plants, after the withdrawal of claims to a patent that had been issued to Lubrizol Corporation of the United States in 1989, which had been challenged by environmentalist protesters.

The Lubrizol patent covered any transgenic plant that had been originally derived from a plant cell transfected with a plant gene under the control of a plant promoter, using a defined gene transfer system.

Officials at the EPO had hoped that the challenge to the patent would be referred by its lower appeals board to its Enlarged Board of Appeals, the office's highest legal authority, which might then reverse a decision made last year — and confirmed by the Enlarged Board of Appeals in December — that plants are in principle not patentable (see *Nature* 374, 8; 1995).

The latter decision concerned a patent that had been granted to the Belgian company Plant Genetics Systems (PGS). It was based on article 53(b) of the European Patent Convention — the EPO's rule book — which explicitly excludes plant varieties from patentability. The appeal board was persuaded by opposition arguments that plants can be considered as constituting plant varieties, and are therefore not patentable.

Following this verdict, whose implications are of major concern to the biotechnology industry, the EPO has not issued any further patents on plants or animals this year. Officials had hoped that a clear verdict in the Lubrizol case would allow them to ask the Enlarged Board of Appeals specifically to clarify the scope of the exclusion article, and therefore perhaps to allow them to lift the moratorium.

But Lubrizol will not after all provide a test case for the patent office. During a hearing on the challenge last week — and before opposition to the patent on the basis of article 53(b) had been heard — the company announced that it was abandoning claims in its patent to the plants and plant cells arising from its transfection procedure.

Lubrizol Corporation said that it was doing this because it could not defend itself against the more conventional criticism of the patent claim that it did not include the required inventive step.

Alison Abbott

Death of molecular biologist stuns San Diego science

San Diego. A prominent Japanese scientist engaged in research into Alzheimer's disease at the University of California at San Diego (UCSD) was murdered virtually on his doorstep on 7 May, sending shockwaves through the city's academic and biotechnology communities.

Tsunao Saitoh, 47, was shot several times in the head as he was returning to his home in the wealthy community of La Jolla. His 13-year-old daughter Loulie, who was wearing a lab coat that may have made her a target, was also shot several times in the attack, shortly before midnight.

Police in San Diego say that they remain baffled over the motivation behind the two murders. Saitoh was known for working 12-hour days in pursuit of a gene expressing a protein that, he believed, was intimately involved in the process by which beta amyloid destroys neurons and causes Alzheimer's disease.

The cold-blooded manner in which Saitoh and his daughter were killed has prompted speculation about whether pressures in the scientific community in San Diego, where many fortunes have been made on the back of the growth of the biotechnology industry, may have contributed to the incident.

In particular, questions have been raised over whether Saitoh's death was related to the race currently taking place to conquer Alzheimer's disease, which in America alone afflicts more than four million people. Some researchers have rejected as implausible the idea that Saitoh's research could be related to his murder. But others point out that violent incidents have, in the past, grown out of personal differences within the research community.

Some years ago, for example, one La Jolla scientist unsuccessfully attempted to poison another after a conflict developed between the two over a research project. A biotechnology venture capitalist points out that fights over patent rights are sometimes known to have become particularly vicious.

Even murder is not unknown. Several years ago, a woman who had testified in court in a patent fight in the San Francisco area was later found strangled, leading to speculation that the murder might have been linked to industrial espionage.

Some laboratories at San Diego institutes are known for their intense competition, with laboratory notebooks disappearing from unsecured lockers, researchers afraid to discuss discoveries at conferences, and the rights to discoveries being misappropriated for the development of biological products.

But the UCSD Alzheimer's Disease

Research Center — which operates under a \$14 million grant from the National Institutes of Health (NIH), and where Saitoh conducted his research — does not have a reputation for such aggressive antics.

Saitoh had been at UCSD for nearly a dozen years, using molecular biology to seek the genetic source of beta amyloid's destructive course in the brain. In addition to the NIH funding, he is said to have also received about \$100,000 a year from the National Alzheimer's Association and a private foundation. He also may have had ties to some biotechnology or pharmaceutical firms.

Saitoh received his doctorate at the University of Kyoto in Japan. Prior to being appointed to his research post in San Diego in 1985, he had carried out research at Columbia University in New York and at the Pasteur Institute in Paris.

During his time in San Diego, he made his mark as a brilliant researcher known also for his teaching skills with young scientists. But acquaintances say that he was discrete about his personal life.

In 1995, he purchased an \$800,000 home near an exclusive private golf club in the heart of the seaside village of La Jolla. He also owned two condominiums in the region. He is estranged from his wife, who was in Europe at the time of the murders. But they reportedly lived in and owned the La Jolla home together.

According to police, at the time of the shooting, Saitoh and his daughter were returning home from his laboratory after working on a school project for the girl. Saitoh was killed at the wheel of his BMW car, while the girl was found some distance away lying on the driveway, indicating that she had been trying to escape when shot in the back and head.

Analysis later revealed that both victims had been struck by shots from a small-calibre handgun. Saitoh's daughter was hit from a distance of about 30 feet, suggesting that the shooting may have been carried out by a professional killer.

In addition to investigating Saitoh's murder, law enforcement authorities in San Diego are also preparing to put on trial a Pennsylvania man involved in an earlier scientist killing, the murder last December of British geophysicist Stanley Runcorn (see *Nature* 378, 657; 1995).

A well-respected scientific figure and a professor at the University of Alaska, Runcorn had been visiting friends in California when he was murdered in a hotel room. Paul B. Cain, a 24-year-old kickboxer who had evaded US authorities for several months by hiding in Mexico, has now been charged with the crime.

Rex Dalton

Brussels urged to back more basic science

Munich. A strong plea for basic research to be given a major role in the European Commission's next five-year Framework programme has come from the European Science and Technology Assembly (ESTA), a 100-member advisory committee set up two years ago with representatives from both industry and the academic community.

In a statement issued last week, ESTA acknowledges that the main role of the commission, which is preparing the first draft of the new Framework programme, to run from 1999 to 2003, is to support applications-oriented research. But it also says the commission should bear in mind industry's need to remain in touch with basic research.

In the past, fundamental research has been considered in Brussels as mainly the responsibility of individual countries. This has been changing in recent years; many research programmes in the current ECU12.3-billion (US\$15.2-billion) fourth

Framework programme, for example, include a basic research element, which tends to be heavily oversubscribed.

In addition, the current research training programme — Training and Mobility of Researchers — is widely used to promote the training of young basic scientists. But many European scientists now fear that the prevailing mood of budget cuts and targeted spending may erode even this small role in the next Framework programme.

In contrast, ESTA argues that support for both training young scientists and basic research should be expanded in the next Framework programme. The former, it says, is essential to provide industry with a highly skilled workforce already experienced in adapting to the international environment.

The assembly also argues that every programme in Framework V should include a basic research component. This would fund 'bottom-up' proposals, allowing researchers to help define how long-term industrial tar-

gets might be reached, and enhancing the benefits from serendipitous discoveries.

In its statement, ESTA says that the commission should develop 'common [scientific] objectives' between national research councils and Europe-wide research bodies, such as the European Southern Observatory, the European Molecular Biology Laboratory, in Heidelberg, and the European Laboratory for Particle Physics (CERN) in Geneva.

Indeed, some ESTA members would like the commission to recognize the expertise of large international laboratories in organizing major research programmes, and to give them responsibility for administering certain programmes.

Jan Borgman, the chairman of ESTA and formerly head of the Dutch National Research Council (NWO), is confident that the assembly's opinion will be taken seriously by the commission, in particular because it is shared by both its academic and industrial members. But whether its suggestions will be accepted will not be known until the first draft of the fifth Framework proposals is published at the end of the year.

Edith Cresson, the research commissioner, is said to be sympathetic to the plea that basic research should not be forgotten. But the Council of Ministers, made up of representatives of member states and facing pressing problems such as unemployment and agricultural policy, may need more convincing. Both the content and funding of the new Framework programme must be approved by both the council and the European Parliament.

ESTA is facing its own internal problems. Having argued unsuccessfully that ESTA needs more administrative help to handle its increasing burden of work — in the two years of its existence, the assembly has set up half a dozen working groups and plans still more — Borgman resigned at the end of March in protest at the workload he was having to carry.

ESTA's bureau, a group of 20 members, failed to agree on a successor last month. Borgman, who continues to hold the reins temporarily, says that he would stay in office if more help were to be provided from Brussels. But this seems unlikely, and a new chairman will probably be selected at the next ESTA assembly in July.

Meanwhile, biologist members of ESTA have agreed to set up an informal group, organized by François Gros, the former director of the Institut Pasteur in Paris, to suggest how the European Union should be targeting its biological research.

According to Gros, biology has become so complex that deciding on future priorities — taking account of the need for future industrial applications — needs wide consultation within industry and the academic community.

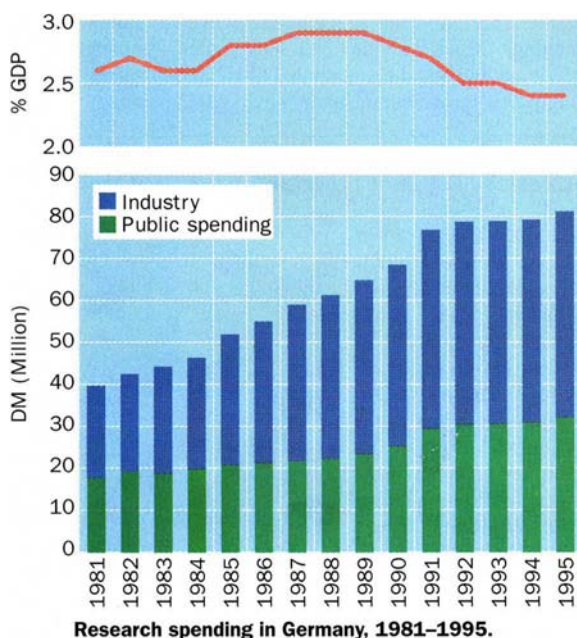
Alison Abbott

German research spending stays low

Munich. The funding of research in Germany over the past three years has not kept up with inflation, while the proportion of domestic research financed by industry has dropped significantly, according to statistics published last week.

In the introduction to a report analysing research and development (R&D) spending, Jürgen Rüttgers, the German research minister, says that high wages, as well as a restrictive and bureaucratic research environment, have forced companies — particularly in the chemical industry — to move

much of their R&D activities abroad. But his Social Democrat (SPD) opponents say that by focusing on industry, Rüttgers is trying to distract attention from the stagnation of public research funding in the past three years. Germany's federal research budget now stands at 2.3 per cent of gross domestic product (GDP), compared with 2.9 per cent in 1989. According to Reinhard Junker, an SPD spokesman on science, the effect is that the federal research budget is "at



Research spending in Germany, 1981-1995.

least DM3 billion (US\$2 billion) short of what it should be".

The Bundesverband der Deutschen Industrie, German industry's umbrella group, has also reacted angrily to Rüttgers' attempts to blame industry for a slowdown in research budgets. "It is no use complaining about decreasing industrial research but still not taking effective measures to ease tax burdens and licensing procedures for R&D departments," says a spokesman.

Quirin Schiermeier

Once upon a smoggy day in London town...

London. The emission of minute airborne smoke particles from motor vehicle exhausts needs to be cut by at least two-thirds if pollution-related illnesses are to be reduced, according to a group of air pollution advisers to the British government.

In its third report, published last week, the 10-member Quality of Urban Air Review Group says that existing measures are "unlikely" to reduce concentrations of the particles below the government's agreed limit of 50 micrograms per cubic metre over a 24-hour average.

Epidemiological evidence suggests an association between high concentrations in the atmosphere of smoke particles smaller than 10 micrometres in diameter — known as PM_{10} — and adverse health. The associated effects include higher than average death rates for heart and lung diseases, as well as a rise in the United Kingdom in the prevalence of allergic disorders such as asthma, hay fever and eczema.

The report, *Airborne Particulate Matter in the United Kingdom*, cites several examples of the 50 microgram limit being exceeded in British cities. For example, in central London, this value was exceeded on 139 days when measured as a 24-hour running mean

Choking: particle limits are being exceeded.

between 1992 and 1994; on 152 days for Belfast and 99 days for central Birmingham.

Vehicle exhaust emissions contribute a quarter of all emissions of primary airborne particles — those emitted directly from the source into the air. This fraction is higher in winter months and in urban areas. Statistics for Greater London in 1990, for example, show that 86 per cent of exhaust emissions by weight consisted of PM_{10} .

In urban areas, most particulate matter from vehicle exhausts tends to be 2.5 micrometres in diameter, particularly in winter. The report adds that emission control

strategies should target sources of PM_{10} that "are well defined and are controllable".

But despite pressure in the United States for $PM_{2.5}$ to become a standard measurement (see *Nature* **380**, 10–12; 1996) — either instead or in addition to PM_{10} — Roy Harrison, chairman of the group and professor of environmental health at the University of Birmingham, says such a change is unlikely in the United Kingdom. "There is a misconception in the press that we are somehow behind the game. But I am quite happy with the standard we have set."

The report puts the government in a difficult position. About four-fifths of road traffic PM_{10} comes from diesel engines. But ministers are unwilling to intervene in the sale of such engines. Instead, they have already agreed to implement technical improvements in diesel technology to cut PM_{10} emissions from new diesel engines.

But further technical improvements, says Harrison, will be less beneficial because a diesel engine modified to emit smaller quantities of PM_{10} emits more nitrogen oxides. A spokesman for the Department of the Environment says the government will study the report closely before announcing its air quality strategy in the summer. **Ehsan Masood**

Advisers deny pressure over Japan's 'first' AIDS case

Tokyo. The failure of the Japanese government and its science advisers to recognize the first case of AIDS in Japan, a haemophiliac who died in July 1983, has become the latest focus of attention in the scandal over the infection of haemophiliacs with HIV in the early to mid-1980s.

This failure is widely believed to have delayed government action to halt the spread of HIV through blood products. Indeed, it has even been suggested that the government deliberately chose not to recognize the case.

Last week, Yuichi Shiokawa, emeritus professor of Jutendo University, who served on the Ministry of Health and Welfare's AIDS study group in 1983 and subsequently headed the government's AIDS Surveillance Committee, testified on the case to a lower house parliamentary committee. This followed testimony by other members of the study group (see *Nature*, **380**, 660; 1996).

The haemophiliac was a patient of Takeshi Abe, former vice president of Teikyo University and head of the AIDS study group, which was set up in June 1983, just before the patient died. Abe presented the patient's records to the group at its first meeting. But the group decided not to recognize the patient as an AIDS victim, even after a US expert told Abe in August 1983 that this was definitely so.

The case was eventually recognized by

the government as AIDS two years later — but only after the AIDS Surveillance Committee, headed by Shiokawa, had announced in March 1985 that Japan's first case of AIDS was a Japanese homosexual who made a temporary visit to Japan from the United States. The homosexual was treated by a colleague of Shiokawa's at Jutendo University on 17 January 1985, and returned to the United States the following day.

Last month, Juzo Matsuda, also of Teikyo University and a former member of the study group, testified that at the first meeting of the study group in June 1983, Shiokawa favoured publicly recognizing Abe's patient as an AIDS case. But he later reversed his position.

Matsuda suggested that Shiokawa's change of heart was brought about by pressure from top ministry officials, or former top ministry officials who had retired to positions with domestic blood product manufacturers. He argued that if the patient had been recognised as an AIDS victim, emergency measures — such as the import of heat-treated products — would have to have

been taken, which could have damaged the domestic blood product industry.

Shiokawa, giving unsworn testimony last week, denied that he was put under any such pressure. He also argued that, as the study group was headed by Abe, he did not have strong influence on its decisions. But Shiokawa did admit that the AIDS case that he announced in March 1985 probably would not be diagnosed as AIDS under current diagnostic criteria.

There is growing evidence that the government deliberately suppressed information about HIV infection among haemophiliacs, and it is being widely suggested in Tokyo that the homosexual case may have been used to mislead the public into thinking that AIDS was only a problem among homosexuals.

Matsuda testified that in late 1984, Abe, who was at the same university as Matsuda, told the ministry of health and welfare about test results from the United States which showed that 23 out of 48 of Abe's haemophiliac patients were HIV positive. But the ministry says it has no record of his findings.

Last month, however, the ministry released documents showing that Shiokawa's surveillance committee was at least informed about four other HIV-positive haemophiliacs in November 1984. But the committee did not make the findings public. **David Swinbanks**



Shiokawa: changed his mind about AIDS case.

US environment projects may merge efforts

Washington. A planning team led by policy advisers in the White House is exploring ways of merging the current patchwork of US government environmental programmes into a single coordinated network for ecological research and monitoring.

The proposed network would go far beyond the problem-ridden Environmental Monitoring and Assessment Program (EMAP) sponsored by the Environmental Protection Agency (EPA), which has been scaled back in recent years (see *Nature* 374, 486; 1995). Instead, it would link dozens of government research projects ranging from water quality research to remote sensing.

The planning effort is being led by the National Science and Technology Council's Committee on Environment and Natural Resources (CENR), which has identified about 30 government programmes that spend roughly \$500 million a year on environmental monitoring and research.

These include the US Geological Survey's extensive network of stream gauges; surveys of breeding birds; monitors that test air quality for compliance with EPA regulations; Forest Service monitoring stations; and a network of 18 long-term ecological research (LTER) sites funded by the National Science Foundation.

According to Rosina Bierbaum, who took over recently as acting head of the CENR from Robert Watson on his move from the White House Office of Science and Technology Policy to the World Bank, there are also many non-federal programmes. "We're just beginning to barely understand what all the monitoring networks might be at the

regional or state or local level," she says.

At present, the networks gather information on different temporal and spatial scales, and their data exist in different formats. "There are a lot of things going on, but they are not well coordinated," says Watson.

The CENR invited scientists and resource managers working in a single region — the mid-Atlantic — to a workshop last month to begin building a conceptual framework for the integrated network. A second, national workshop is planned for

making existing programmes more compatible with each other.

Many sites could probably be adapted or augmented to collect additional data. But, says Watson, "we almost certainly will need some new [monitoring] sites". The integrated network may take years to develop.

The EPA's EMAP programme, once viewed as a grand-scale nationwide ecological monitoring programme, would be part of the network. Robert Huggett, recently appointed chief of science at EPA — and a critic of EMAP before taking his new job — is co-chair of CENR's team for creating the new integrated network.

N. Smith/Hutchison

Another agency to figure in the plan is the now disbanded National Biological Service (NBS), which by October will be reincarnated as the Biological Resources Division of the US Geological Survey, headed by a new 'chief biologist'. Ronald Pulliam, currently director of the NBS, says he is not interested in the job.

Persuading government agencies to work together will be the most difficult part of creating any integrated network. Watson says he does not foresee one agency taking charge of the programme. "As soon as you give an agency lead responsibility, all the other agencies walk away from it, or you end up with a war," he says.

Ensuring data compatibility among the various projects will be of paramount importance. Remote-sensing data, which will be flooding in from a whole new generation of Earth-orbiting satellites, will have to be made "user-friendly" to be of value to mainstream ecologists.

"A lot of thought needs to be given to exactly what [data] you try to store and how," says Gordon Orians of the University of Washington in Seattle, president of the Ecological Society of America. "There's a tremendous amount of hype on all this electronic [data] storage. But unless it's carefully done and there's a lot of quality control, I just don't think it will be used that much."

Nevertheless, Orians supports the CENR effort, and is cautiously optimistic it will succeed. James Gosz of the University of Mexico, who chairs the LTER executive committee and once headed the National Research Council panel that evaluated EMAP, also supports the idea of an integrated network. But, he says, "it's not going to be easy" when agencies feel their own programmes are being threatened.

Gosz believes the CENR planning workshops are the welcome beginning of a major government effort in wide-scale ecological monitoring. Such a coordinated research effort would be comparable to the Global Change Research Program, he says — "only bigger".

Tony Reichhardt

Chesapeake Bay: test-bed for new approaches to the collection of information on natural resources.

the summer. "We've already got a lot of the agencies thinking very hard about their monitoring systems and how to coordinate them better," says Bierbaum.

According to Watson, the first step is to identify what scientific questions need to be answered. He says the plan does not assume that more money will be available for environmental monitoring, but rather that the government can squeeze more value from its current \$500-million investment by

'Indian ginseng' brings royalties for tribe

New Delhi. An indigenous Indian tribe has been awarded the intellectual property rights to the active ingredient of a plant long known to it as helping to combat stress, in a move that the government hopes will help end the 'piracy' of tribal knowledge by both Indian and foreign drug companies.

The drug *jeevani*, which is based on this ingredient and is said also to provide an instant source of energy, has been developed from the plant *Trichopus zeylinicus* by the government-owned Tropical Botanical Garden and Research Institute (TBGRI) in Trivandrum, the capital of Kerala state.

After successful clinical trials, the institute has transferred the manufacturing know-how to Arya Vaidhya Pharmacy (AVP), a large manufacturer of ayurvedic drugs — drugs based on traditional Indian herbal medicines — for \$50,000.

The Kani tribe of the Agasthiyar hills in the southern state of Kerala will receive half of the know-how fee, and will also receive a share of a two per cent royalty on any future

drug sales. This money will go towards 2,500 families of the Kani tribe who will cultivate and supply the plants to AVP at a price agreed with the TBGRI.

"The world did not know about this unique plant until the Kani people led us to it," says Palupu Pushpangadan, director of TBGRI. His institute started research on the plant seven years ago, after researchers noticed that the tribe members habitually ate its raw seeds before undertaking strenuous work.

Research at TBGRI concluded that the plant contained substances that stimulate the immune system. "Our *jeevani* acts like Chinese ginseng," says Pushpangadan. "But it is superior, because it does not contain any steroid." Extensive trials of the herbal product were carried out on Indian athletes before the institute decided to market it.

A spokesman for the drug company, which plans to market *jeevani* internationally as a rival to ginseng, claims that it is a "money-spinner".

K. S. Jayaraman

'Science and society' course is under threat at Stanford

Washington. Stanford University in California may have to stop awarding degrees in Science, Technology and Society (STS), following a decision by the university's school of engineering that it cannot continue to run the programme in its present form.

The crisis at Stanford has alarmed STS specialists across the United States, who feel that it is representative of attacks on their discipline from scientists who doubt its value. But faculty members at Stanford say that the problem there results chiefly from the difficulty of defending interdisciplinary work at a time of financial cutbacks.

The engineering school said last month that it would stop awarding degrees with STS as their major component in 1997, although it has since agreed to delay the change until 1998.

The basic problem with the programme, says John Bravman, associate dean of the school, is that not enough senior faculty members are prepared to devote time to leading it. "We couldn't find a senior member of faculty to chair it," says Bravman. No-one questions the validity or importance of the programme, he says, but staff have other important things to do. "People really voted with their feet," he says.

Observers of the situation at Stanford say that the problems faced by the STS programme reflect a common difficulty with interdisciplinary programmes: when it comes to the crunch, specialist disciplines will always take priority.

"It was not hostility to STS but indifference and lack of an incentive structure that awards interdisciplinary work that caused our problems here," says Paul Edwards, an assistant professor on the programme.

The STS programme at Stanford has had a troubled history since it began in 1971. Originally controlled directly by a university vice-provost, the programme was given to the Science and Humanities school when that post was eliminated. It was transferred to the engineering school in 1992, partly to ensure greater technical input.

The programme has traditionally concentrated on undergraduate teaching. Its largest activity delivers STS courses to undergraduates who will major in science or engineering, but 50 undergraduates are on track to major in STS, and Stanford plans to stop this option after they have graduated.

A major effort by about thirty faculty members last year led to a detailed plan to extend the programme to include graduate teaching and research. But the expansion plan was rejected by the university administration, partly because of its cost and partly because of a belief that the faculty would not be prepared to provide adequate support.

STS has recently come under fire from scientists who have disparaged the ability of social scientists to mount any useful assessment of science (see, for example, *Nature* 375, 439; 1995).

Such criticisms have not been prominent at Stanford. But some students feel that their course is a victim of a wider backlash against social science. "Programmes across



the country are having problems," says Michael Putnam, an STS student at Stanford. "Engineers don't necessarily like people taking a critical view of technology."

Trevor Pinch, chair of one of the strongest STS departments in the country at Cornell University in New York state, admits that "some of the debate has been very ill-considered". But he says that most senior science faculty respect and support STS, even if they "don't share our view of the world".

The discipline won full departmental status at Cornell five years ago — the first new department there since computer science twenty years earlier — enabling it to confer its own degrees, and thus shielding it from some of Stanford's problems.

Meanwhile, students at Stanford are fighting hard to defend the STS programme, and will discuss its future with the curriculum committee of the School of Humanities and Sciences at Stanford this week. This will lead to what could be a decisive meeting of the university senate in two weeks.

According to several officials, the STS programme may still be saved. "The question is whether a case can be made that is so compelling that they have to keep it," says Robert McGinn, acting chair of the programme. "That debate has still to run its course. I am still hoping for a favourable outcome." Gerhard Casper, the president of Stanford, told students last week that the question of continuing with degrees in STS was "still open".

Colin Macilwain

French cancer charity introduces new rules to restore probity

Paris. France's biggest medical research charity, l'Association pour la Recherche contre le Cancer (ARC), last week sought to put behind it on the financial scandal that rocked the organization earlier this year. A meeting of its general assembly overwhelmingly approved a purge of the executive board and the introduction of a series of checks and balances to ensure that donations are spent on high-quality research.

ARC's continued existence has been in doubt ever since the national audit commission confirmed allegations that only a quarter of the charity's spending went to research (see *Nature* 379, 103; 1996). The audit commission also found evidence of fraud in the operations of companies associated with ARC.

The measures adopted last week are aimed at ensuring the charity's survival by rebuilding public confidence in its activities. The decision to oust 21 of the 26 members of the executive board is an essential step towards this goal, according to Michel Lucas, who replaced Jacques Crozemarie as chairman of ARC after the scandal.

Indeed, Lucas himself was elected chairman in the belief that he would be seen by the public as being above suspicion, as the former head of a government agency that had fought for more than a decade to expose financial irregularities at the charity (see *Nature* 397, 385; 1995).

At the same time, a purge of the existing board was considered necessary because many of its members had endorsed ARC's past practices and had defended the charity against earlier allegations similar to those eventually confirmed by the audit commission.

Those ousted include Dominique Bellet, director of the immunochemistry laboratory at the Institut Gustave Roussy near Paris, which had received the single largest ARC grant in 1993, FF8 million (US\$XX million). To prevent such conflicts of interest recurring, the general assembly also ruled that in future board members would be prohibited from receiving ARC grants.

Last week's meeting represents a watershed in the management of ARC. But a broader impact has come from the public spotlight that the affair has thrown on the glaring lack of regulation of charities in France. For example, the government has for the first time given its own agencies broad powers to investigate charities, while charities themselves are agreeing to follow charters of good conduct. They have good reason; one fallout from the scandal at ARC has been a sharp drop in donations, not only to ARC but also to other medical research charities.

Declan Butler

Genetic testing outside research acceptable, says cancer society

Washington. The American Society of Clinical Oncology (ASCO) has split with other prominent biomedical research bodies by announcing in the May issue of the *Journal of Clinical Oncology* that genetic testing need not be confined to research settings.

The society, whose 10,000 members are all cancer specialists, says that testing for mutations predisposing to cancer should be made available to selected patients "as part of the preventive oncologic care of families". ASCO says that testing should be performed "to the greatest extent possible" as part of long-term outcome studies. But, it adds, "all individuals at hereditary risk for cancer should have access to appropriate genetic testing".

In contrast, organizations such as the National Advisory Council for Human Genome Research, the American Society of Human Genetics, and the National Breast Cancer Coalition have urged that genetic testing be confined to research settings. Frances Visco, for example, the president of the National Breast Cancer Coalition, says in the same journal that breast cancer advocates need the medical community to recognize "the need to do less, rather than more". □

More vaccine research urged

London. Although new viruses such as Ebola, Hanta and Dangué have emerged with alarming regularity in recent few years, little is being spent on vaccine research, according to Donald A. Henderson, director of the Smallpox Eradication Campaign at the World Health Organization. Mankind's survival in an increasingly populated and well-travelled world requires that funds be made available rapidly to develop vaccines against emerging viruses, he said last week.

Addressing a meeting at London's Royal Society held on the

bicentenary of the first use by Edward Jenner of cowpox as a vaccine against smallpox, Henderson pointed out that vaccines were most needed in poorer countries, but that few national research authorities in the developed world were willing to support medical research which does not directly benefit their own citizens. "Resources are not the problem; it is our priorities which are," he said. □

Neuroscience facilities opened

London. Representing one of the most important investments in British neuroscience for many years, the Wellcome Department of Cognitive Neurology and the Leopold Muller Functional Imaging Laboratory in London were officially opened last month by Sir Andrew Huxley, the physiologist and Nobel laureate.

The building, part of the Institute of Neurology in London's Queen's Square, has been financed through a £20-million (US\$30-million) grant from the Wellcome Trust. The Leopold Functional Imaging Laboratory is supported by a £3.6-million endowment from the trustees of the Leopold Muller Estate. □

New fellowships for Stanford

San Francisco. In an effort to reduce its dependence on federal funding and remain attractive to good graduate students, Stanford University in California has announced that it is to fund up to 300 graduate fellowships in the sciences and engineering. The graduate fellowship programme, launched with a \$10-million start-up fund, is intended to free students to pursue their interests at Stanford without the need to rely on federal research grants.

Gerhard Casper, president of Stanford, says he hopes to raise \$200 million to sustain the graduate fellowships, which would equal roughly half Stanford's federally funded research assistantships.

According to Charles Kruger, vice-provost and dean of research and graduate policy, the fellowship programme is intended to



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Impact factors can mislead

SIR — Impact factors (IFs) for scientific journals, developed by the Institute for Scientific Information (ISI) and published in the section "Journals per category, ranked by Impact Factor" of the *Journal Citation Reports (JCR)*, are frequently used to evaluate the status of scientific journals or even the publication output of scientists. The IF of a journal in year T is defined as the number of citations in year T to documents published in that journal in years $T-1$ and $T-2$, divided by the number of

purchased from ISI. In each category we compared the ranking of journals by IF as printed in the *JCR* to the one based on our correct IF, by calculating the number of journals moving at least 1, 3, 5 or 10 positions. The table shows the five categories affected most severely, measured through the percentage of journals moving at least one position in the ranking. The categories listed relate not only to the medical sciences but also to chemistry and engineering sciences. The percentage of journals dropping

FIVE CATEGORIES SHOWING LARGEST DIFFERENCES IN RANKINGS BY IF PRINTED IN ISI'S *JCR* AND BY CORRECT IMPACT FACTOR

Category	No. journals	% Journals with rank difference			
		≥ 1	≥ 3	≥ 5	≥ 10
Chemistry	130	89.2	40.8	6.9	4.6
Medicine, general and internal	152	80.3	32.2	11.2	3.9
Pharmacology and pharmacy	171	78.4	25.7	8.2	3.5
Medicine, research and experimental	86	76.7	14.0	7.0	2.3
Engineering, electrical and electronic	114	70.2	18.4	6.1	3.5

citeable documents published in that journal in years $T-1$ and $T-2$. But the concept of citeable document is not defined accurately by ISI.

Eugene Garfield has pointed out that, for 40 leading medical periodicals, journals differ with respect to the numbers and types of documents they publish, and variations exist in impact for different types¹. We obtained evidence that the IFs of many journals included in the *Science Citation Index (SCI)* are inaccurate because of an inappropriate definition of citeable documents².

ISI classifies documents into types. In calculating the nominator of the IF, ISI counts citations to all types of documents, whereas as citeable documents in the denominator ISI includes as a standard only normal articles, notes and reviews. However, editorials, letters and several other types are cited rather frequently in a number of journals. When they are cited, these types do contribute to the citation counts in the IF's numerator, but are not included in the denominator. In a sense, the citations to these documents are 'for free'. For instance, taking into account only citations to normal articles, notes and reviews in *The Lancet*, the 'correct' impact factor of this journal in 1992 would be 43 per cent lower than the IF listed in the *JCR*.

We calculated for each *SCI* journal a 'correct' IF for the year 1994 by taking into account in the IF's numerator only citations to articles, notes and reviews, and including in the denominator the same three types. We used a special datafile

Among these, there is a substantial number of 'top' journals.

Our analyses suggest that journal editors or scientific publishers could, in principle, artificially raise the IFs of their journals. To put it bluntly, if a scientific publisher succeeds in publishing important review articles

as an editorial, or including a lively correspondence section, the IF of his or her journal may go up substantially. Moreover, scientists whose publication output is weighted by the IFs printed in the *JCR* may see their scores descend when correct IFs are used.

Evaluators of scientific output or journal performance should be cautious in using the impact factors printed in ISI's *Journal Citation Reports*.

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1. Garfield, E. *Current Contents*, 12 January 1987.
2. Moed, H. F. & van Leeuwen, Th. N. *J. Am. Soc. Inf. Sci.* **46**, 461-467 (1995).

Failed professor

SIR — Your journal has shown itself sensitive about Spanish scientific policy and assessment of and awards to university professors and researchers.

The present system of marking and assessment, based only on the *Science Citation Index (SCI)* of the *Journal Citation Reports*, is unfair to those who have contributed to international journals but apparently not the right ones.

Nobody doing research in Spain at the beginning of the 1980s could foresee that a list of journals compiled by the *SCI* was

going to determine whether one's research was 'valid' or not. Research productivity is now evaluated by a National Committee selected by the Spanish Science and Education Ministry, which simply rates one's research according to the number of *SCI* publications accumulated over a six-year period.

This may work in some fields, but it fails in botany, geology and zoology, for example, because these disciplines do not generate as many articles or citations as, say, biotechnology or genetics. The combination of less frequent citations and a publishing market now saturated with new journals renders the *SCI* estimated impact factor of these publications close to zero. Many titles are never indexed by the *SCI*.

I am a graduate zoologist, doctor and university professor who was failed by a National Committee which, it seems, takes account only of the *SCI* ratings. It is ironic never to have failed an examination as an undergraduate, to have been awarded a doctorate in zoology and to have obtained a professorship and then to have been failed by a National Committee. Despite a research track record judged by editorial boards, granting agencies and international colleagues to have been active and productive, I was graded 4 on a scale of 10.

There is considerable room for improvement in the way the system operates.

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Slow, slow...

SIR — You perpetuate the myth that HTTP is inherently slower than FTP for single file transfers (*Nature* **380**, 380; 1996). This is patently untrue; both protocols relegate the responsibility for file transfer to a lower level protocol, TCP (transmission control protocol), identical in both cases. In fact, HTTP is marginally more efficiently designed than FTP as it transfers the file down the same connection as the request, whereas FTP opens an entirely new connection for the file transfer.

So, if it can be experimentally verified that an HTTP is indeed slower than FTP (a fact often claimed but rarely supported by any evidence) one must conclude that the difference is caused by poor implementation of the protocol, either by the client or the server, or both.

Of course, HTTP is poorly designed in other ways, particularly when used to transfer modern Web pages made up from many files, as the article correctly points out.

Ben Laurie

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An economic case for basic research

Eugene Wong

Contrary to the anecdotal evidence of the 1980s, basic research does confer a preferential economic advantage on countries that fund it. That is why the United States is likely to dominate vital markets well into the next century.

EVERYWHERE in the industrial West there are pressures to reduce public funding for basic research in favour of more applied work. In part, the pressures are due to a change in the public perception of the benefits of basic research. But even those who understand the value of basic research often question whether it confers any special economic advantage on the country that funds it.

Whenever the issue is debated, Japan is cited as an example of a country that has benefited from basic research without funding very much of it. Instead, Japan has emphasized product development and high-quality manufacturing. In so doing, it had captured, by the late 1980s, one after another of the science-based markets: television sets, video recorders, liquid-crystal displays and dynamic random access memories (DRAM) — all technologies invented and commercialized by the United States. The loss of the DRAM market was particularly disturbing. Because this part of the semiconductor market requires the most sophisticated manufacturing technology, it was widely predicted that the loss of the DRAM market would lead to the atrophy of the entire US semiconductor industry. The perceived loss of competitiveness by precisely those countries that have done the most to fund basic research has undermined its public support.

Japan's example has been successfully followed by other economies of the Asia-Pacific region. Singapore has attained the status of a developed country in one generation, and its economy is growing three times faster than that of the United States. It has achieved this without funding much — if any — basic research. Hong Kong, another tiger economy of the region, is starting to build an infrastructure for research. After several years of rapid development, its total research spending amounts to 0.03 per cent of gross domestic product (GDP), less than a tenth of the percentage spent by the United States. In Asia it is often said that "research is a luxury that only rich countries can afford".

The science and technology policy of the United States in the past 50 years was shaped largely by the experience of the Second World War, in which science had a

decisive role. The tenets of the policy are set forth in the 1945 report by Vannevar Bush, *Science — The Endless Frontier*¹. First, science must be called upon to serve some of the most critical of national needs. Second, public funding for research is a vital government function. Third, the benefits of science should be

most people in the world.

With Japan and the Asia-Pacific region as apparent counter-examples, two specific aspects of the Bush model have come under question: its emphasis on basic research and its reliance on diffusion as the primary way of capturing the benefits of science. Some call for a more proactive government policy that would give greater weight to applied work and make a more deliberate effort to select areas of research for their commercial promise.

Steve Dunwell/TIB

Although it is premature to assess fully the validity of these challenges to the Bush model, there are recent indications that the evidence on which they are based is not what it seems.

Science deals with knowledge, whereas technology is the means by which knowledge is applied. The distinction between these concepts is both significant and clear. But the distinction between basic and applied research, or between applied research and development, is less clear. Basic research is here defined as activities that lead to fundamental understanding and discoveries, whether motivated by applications or by mere curiosity.

The forces of a free market are an excellent allocator of investment funds. What then is the justification for government investment in research and development (R&D) that will ultimately lead to commercial products? Why not simply reduce taxes and let industry make the investment in R&D? A persuasive answer lies in appropriability of returns.

Some activities in R&D produce large social returns: that is, returns that benefit society at large but provide little or no preferential gains to those who make the investment. That being the case, there is inadequate incentive for private companies to make such investments. Basic research is a case in point. In the aggregate, basic research has provided enormous economic returns², but these returns are social, not private. For example, the discovery of quantum mechanics, among other things, was responsible for the eventual development of the entire modern electronics industry. But even with the benefit of hindsight, quantum mechanics would not have been a good private investment.

Lucky chips: Japan captured an important part of the computer industry by acquiring and using basic knowledge rather than producing it.

allowed to diffuse through natural market mechanisms.

The policy formulated by Bush was implemented with remarkable success in the ensuing years. Federal funding of research and development expanded vastly, and with it a research establishment of extraordinary productivity and creativity. From health care to defence, the unique role of science in serving national needs is unquestioned. The contribution of science to the post-war economy of the United States, indeed the world, is particularly important. For example, microelectronics, an entirely post-war industry that has depended on science for every step of its development, now touches the daily lives of

Given its large returns and lack of appropriability, justification of the public role in funding basic research is seldom questioned. What is questioned, however, is whether it confers a preferential economic advantage on the country that funds it. If the benefits of research are not appropriable for companies, why should they be appropriable for countries?

Japan is the model of an advanced industrial country that has specialized in acquiring and using the results of basic research rather than doing it. Japan's total investment in R&D as a percentage of GDP (2.9 per cent as against 2.7 per cent in the United States) leads the world. The private sector share of the investment is truly impressive (81 per cent of total versus 53 per cent for the United States)³. Indeed, with a substantially smaller GDP, the Japanese private sector investment in R&D exceeds that of the United States in absolute terms. When one adjusts for the much larger portion of R&D spending devoted to defence in the United States, the difference is even more striking. But very little of Japan's R&D, public or private, is basic research.

Japan invests more not only in R&D, but also in total. The investment rate as a percentage of GDP is nearly twice as high in Japan as in the United States⁴ and the gap is not closing. Over the decade 1980-90, nonresidential capital investment in Japan rose from 15 per cent of gross national product a year to 19 per cent, while in the United States it declined from 13 per cent to 10 per cent⁵. More input should yield more output. Indeed, the increase should accelerate⁶.

Input is made up of labour and capital. Because changes in the labour component are small⁶, Japan, with twice the capital investment rate, should have more than twice the rate of economic growth. But that is not the case. The high rate of growth in Japan in earlier years has slowed dramatically. Even after it recovers from its current recession, the sustainable rate of growth in Japan is estimated to be no more than 3 per cent of GDP a year⁴, whereas the comparable estimate for the United States is 2.5 per cent⁷. So despite its greater R&D investment, Japan's economy is considerably less, not more, efficient. If acquisition of research results is a superior technology policy, economic data do not show it.

The comparison with Japan also applies to other countries of the Pacific Rim. Singapore, for example, has three times the growth, but only by investing four or five times as much⁴.

In looking for areas where the United States and Japan have large differences, one has to consider their university and research infrastructures. Universities and

basic research in the United States have been major beneficiaries of its post-war science policy. By any reasonable measure, they have reached heights never previously scaled. Their superiority over their counterparts in Japan cannot be questioned. It is a reasonable hypothesis that this superiority has contributed in important ways to the efficiency of the US economy.

The United States is not the only coun-

'Video streams' — film frames spread out like a pack of cards — are one of a range of revolutionary media technologies being developed in the Media Lab at the Massachusetts Institute of Technology.

try to have benefited from a commitment to research and higher education. Europe continues to reap the rewards for its support of basic research and universities over the past century or more. It is no accident that two of the industries most dependent on research, chemical and pharmaceutical, continue to flourish in Europe. The smaller communities in Asia, such as Hong Kong and Singapore, should consider the case of Switzerland. It has managed to retain a healthy share of manufacturing in its economic mix, despite some of the highest wages in the world and without government intervention. Its success is due in no small part to its outstanding universities and its unwavering commitment to high-quality research.

Although the evidence on the role of the universities and importance of basic research is not conclusive (economic evidence rarely is), there is no evidence at all to support the alternative hypothesis that reducing public support for universities or de-emphasizing basic research would be a wise policy.

As the economist Paul Krugman observes⁷, the real miracle of Asia is the ability of the countries in the region to produce sufficient savings to finance capital investments at 20, 30, even 40 per cent or more of GDP while improving their standards of living. In savings and investment at least, these are no paper tigers.

Meanwhile, the industrial nations of the West are straining to invest even 10 per cent of GDP. The culprit in almost every case is medical costs, which in the United States now represent nearly 15 per cent of GDP, and are rising. By contrast, the total funding for basic research, both public and private, is less than half a per cent of GDP (one year's increase in medical costs) and threatened with reduction. Health care is almost pure consumption with a powerful political base. Basic research is almost pure investment with a constituency largely of the unborn. Who speaks for them?

The anecdotal evidence of the 1980s needs updating. The US semiconductor industry did not waste away, and while policy pundits were mourning the loss of the consumer electronics market, US researchers were creating revolutionary markets as diverse as biotechnology, multi-media, computer software and digital communications. None of these would have existed without federally supported research, and none would have existed without the direct participation of some of those who did the research. (Herein lies the secret of appropriability.) As a result, US industry is now in a position to dominate these vital areas of the economy well into the next century. In this regard, Wall Street is a better forecaster than Main Street and Pennsylvania Avenue.

One major barrier to entry into new markets is the requirement to see the future with clarity. It has been said that to foretell the future, one has to invent it. To be able to invent the future is the dividend that basic research pays. □

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1. Bush, V. *Science — The Endless Frontier* (US National Science Foundation, Washington DC, 1945; reprinted 1960 and 1980).
2. Mansfield, E. in *Science and Technology Policy Year Book* (American Association for the Advancement of Science, Washington DC, 1991).
3. Arima, A. *Promoting Scientific Creativity in Japan* (Paper presented at the Conference on Nurturing Creativity in Research, Canberra, 26-28 November 1995).
4. Krugman, P. *Foreign Affairs* (November/December 1994).
5. Landau, R. in *Technology and Economics* (National Academy of Engineering, Washington DC, 1991).
6. Boskin, M. J. & Lau, L. J. in *Technology and Economics* (National Academy of Engineering, Washington DC, 1991).
7. *Wall Street Journal* Sect. A, 6 (11 August 1995).

Sexual conflict as fuel for evolution

Tracey Chapman and Linda Partridge

An experiment with fruitflies, in which females were not allowed to adapt genetically to male change, has unmasked the rapid evolutionary dynamics underlying an ostensibly static sexual system.

MALES and females have a common interest in the production of offspring but, even so, all is not sweetness and light. From the original difference between males and females in the size of sperm and egg cells, many others have followed. Harassing, philandering, infanticidal males, and reluctant, picky, demanding females show that natural selection can result in sexual conflict¹. Coercion by males for matings with reluctant females is common, as are philandering males who abandon their partners with dependent young in favour of new mates. Females, too, can insist on mating only with males offering large enough rewards, and will cuckold their mates for their own gain. The consequences of sexual conflict can be drastic, including infanticide of a female's offspring by a new male and sexual cannibalism.

On page 232 of this issue² William Rice reports the results of an ingenious experiment in which he stopped one side of the sexual arms race in the fruitfly *Drosophila*. When males were allowed to evolve against females that could not retaliate, they rapidly produced a suite of reproductive traits that were inimical to the reproductive success of the hamstrung females. Locked together by their need for partners in sexual reproduction, the sexes undergo an antagonistic dance to the music of time.

The 'adapting' males in Rice's experiment were mated in each generation to 'target' females. The reproductive contribution of the adapting males to the next generation was determined by their success in mating with and producing offspring from these target females. The genetic material contributed by the target females entered the adapting male line afresh in each generation and was discarded at the end of it (see Fig. 1). The adapting genetic material in the males was handed down intact.

Drosophila is the ideal organism for this kind of experiment, because mutant stocks have been developed that make it possible to transmit large parts of the genome intact between generations³. Otherwise genetic recombination would produce chromosomes with mixtures of genes from male and female parents. The adapting genetic material was only ever transmitted by males, because all the female progeny in the adapting male line

were discarded. The target females could not evolve in response to the adapting males because the supply of target females moved down a one-way street — no female that had mated with an adapting male was ever returned to the target female line.

After only 41 generations of selection, the adapting males did much better than the control males (unselected flies from

higher death rates when exposed to adapting males than to controls. Part of the reason seemed to be that the adapting males managed to mate with the target females more frequently (higher mating rates increase female mortality⁴). In addition, matings with experimental males were more dangerous for females than were matings with controls.

These results raise a lot of questions. The target females may not have been able to make an evolutionary response to the adapting males, but they could respond to their own target males with which they were continually reproducing. Why was this not enough to protect them against the adapting males? It could be that the pace of evolution was faster in the adapting males than in the males from the target female line — the adapting males had greater opportunities for genetic recombination, which can speed evolution^{5,6}, and they may have been more genetically variable because they had recently been introduced to the laboratory from the wild⁷.

Alternatively, the males from the two lines may have evolved in different directions, with the females responding specifically to their own males. It would be interesting to know the levels of male reproductive success and female damage-limitation when males of the adapting male, control and target female lines are tested against control and target females. If the adapting males really have evolved to the static female phenotype, then their advantage over control males should disappear or diminish if both types of males are tested with control females.

What of the mechanisms of male adaptation and female counter-adaptation? As to male adaptation, components of the male seminal fluid, which play a role in sperm competition^{8,9} by removing, destroying or incapacitating the stored sperm of previous mates, may be important in determining the higher fitness of the adapting males. Such components are also involved in the induction of egg-laying and reduction of female receptivity to subsequent matings¹⁰, and some of them also show rapid evolutionary change¹¹. They are additionally responsible for the increased death rate in females that remate more frequently¹².

The nature of female counter-adaptation is more mysterious, though it could

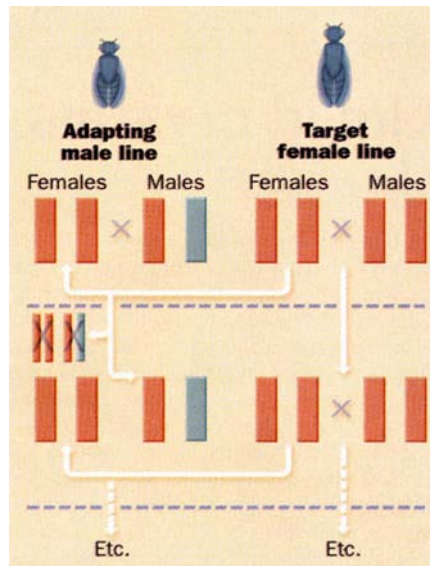


FIG. 1 Rice's protocol simplified, showing two generations only. Blue bars are 'adapting' male chromosomes that were allowed to evolve; red bars are 'target' chromosomes that were not. Adapting males were crossed to new target females every generation, the new target female chromosomes being removed from the adapting male line in the following generation. The upshot of these experiments was the observation that, if one side in the sexual arms race is not allowed to compete, the other (in this case the adapting males) rapidly wins an advantage.

the same base stock as the adapting males, and kept at the same population size as them) with the target females. They produced more male progeny, were better at obtaining matings with already-mated females and were more capable of preventing the females they had mated with from producing offspring by later mates. These are all important reproductive traits that contribute to male fitness — the number of genes passed on to future generations. But these advances were made at the expense of the target females, which had



FIG. 2 Fit for competition? A *Drosophila* egg emerges into the outside world. (Picture in false colour.)

be that hitherto unidentified receptors for seminal-fluid molecules are somehow involved. Sex-limited traits may offer further explanations. For instance, only male *Drosophila* have sex combs on their legs and only females have ovipositors. The genes responsible for sex-limited characteristics such as these are present in both sexes but silent in one. A novel genetic variant producing a molecule in the male seminal fluid that induced females to delay remating would spread by natural selection in favour of the novel males, irrespective of whether it caused premature death in the females, and irrespective of its silent transmission through them. Any female counter-adaptation would most likely result from selection for new variants of quite different genes, such as those for receptors. In a sexual arms race, there are presumably sequential waves of natural selection on different gene loci with sex-limited expression.

Genes can have sexually antagonistic effects by a quite different mechanism, which may bear on Rice's experimental findings. Sexually antagonistic genes are ones that are expressed in both sexes, but where different variants are favoured in males and in females. Their passage to and fro between the sexes over the generations means that the sexes hold back

each other's evolution. We do not know the identity of these genes, but evidence of their existence has come from Rice's earlier work¹³.

In that study, Rice arranged for particular groups of genes to be confined to females, for 29 generations. The idea was that, if selection favoured different variants of these genes in males and females, then releasing them from selection in males would allow female-advantage variants to spread. Again, the process was remarkably rapid. After selection, males carrying the genes that had been previously confined to females were markedly impaired in their fitness relative to controls. In the new experiments², the adapting chromosomes in the adapting male line were mostly confined to males during selection, which may have allowed male-advantage variants to spread. Sexual conflict and sexual antagonism may therefore have put the target

females in double jeopardy. It would be interesting to know what happens when adapting male genomes are expressed in females.

The idea that evolutionary arms races — between parasites and their hosts, predators and their prey, and even between parents and offspring — might produce continuous evolutionary change has been around for a while, but there have been few convincing demonstrations of the principle. Rice's findings provide evidence that sexual conflict may be a potent fuel for evolution, and that in an apparently unchanging sexual interaction males and females may be running frantically to stand still. □

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TECHNOLOGY

Liquid crystals tilted by light

Jos van Haaren

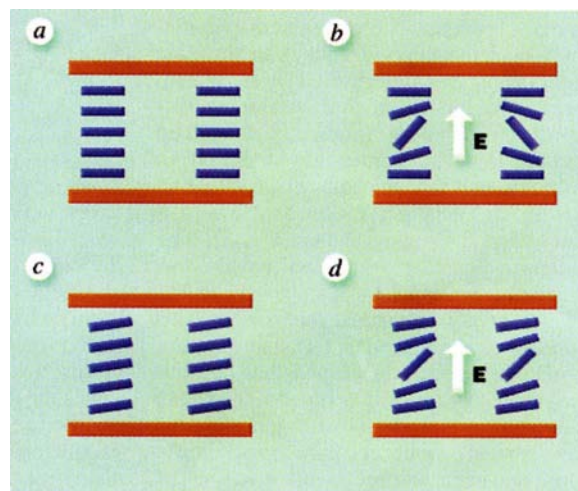
LIQUID-CRYSTAL displays appear in a wide variety of products, from simple segmented devices in watches to colour televisions and electronic work stations. Control over the orientation of the molecules in liquid-crystalline layers is a key topic in the production of these displays. In almost every liquid-crystal display made today, orientation control is achieved with the aid of thin (100 nm) polymer films that are given some directionality by being rubbed with a velvet cloth. On page 212 of this issue¹, Schadt, Seiberle and Schuster report a breakthrough in rubbingless alignment methods.

Their results open the way for replacing the conventional process by a technique in which thin polymer films are illuminated with polarized ultraviolet light. More complex liquid-crystal structures could be made by combining photolithography with this photoalignment technique. This may lead to clearly visible improvements in the quality of displayed images.

To appreciate the relevance of the new results, some background information is needed. Liquid crystals show a natural tendency to align themselves because of the anisotropy in the shape of the molecules, but without any help this alignment is

limited to microscopic domains. It is essential for the operation of liquid-crystal displays that the macroscopic orientation can be externally controlled. The rubbed substrates that sandwich a liquid-crystal element determine the average orientation of the molecular layer next to them; in between, in the bulk of the crystal (typically 5 μm thick), orientation depends on the elastic properties of the material.

Most displays have an orientation that is twisted about an axis normal to the substrate², but a nontwisted configuration will be used for the present argument. All local orientations in such a device (obtained by averaging over a large



Molecular orientations in a liquid-crystal cell. a, b, With zero surface tilt, an applied electric field can produce either arrangement shown in b. c, d, With bias tilt, only one configuration is produced.

1. Trivers, R. L. in *Sexual Selection and the Descent of Man* (ed. Campbell, B.) 136–179 (Aldine, Chicago, 1972).
2. Rice, W. R. *Nature* **381**, 232–234 (1996).
3. Ashburner, M. *Drosophila: A Laboratory Handbook* (Cold Spring Harbor Laboratory Press, New York, 1989).
4. Fowler, K. & Partridge, L. *Nature* **338**, 760–761 (1989).
5. Crow, J. F. & Kimura, M. *An Introduction to Population Genetics Theory* (Harper & Row, New York, 1970).
6. Kondrashov, A. S. *Nature* **336**, 435–440 (1988).
7. Briscoe, D. A. et al. *Conserv. Biol.* **6**, 416–425 (1992).
8. Clark, A. G. et al. *Genetics* **139**, 189–201 (1995).
9. Harshman, L. G. & Prout, T. *Evolution* **48**, 758–766 (1994).
10. Chen, P. S. et al. *Cell* **54**, 291–298 (1988).
11. Aguade, M. et al. *Genetics* **132**, 755–770 (1992).
12. Chapman, T. et al. *Nature* **373**, 241–244 (1995).
13. Rice, W. R. *Science* **256**, 1436–1439 (1992).

number of molecules) are initially parallel to the substrates (part *a* in the figure). Transparent electrodes are used to apply an electric field normal to the substrates. The field induces a dipole preferentially along the long axis of the aligned molecules, and drives them towards an upright orientation. This can be described as the effect of an anisotropy in the material's dielectric permittivity. Similarly, an anisotropy in refractive index translates the changing alignment into an optical modulation when observed between polarizers.

The molecules can fall into either of the arrangements in part *b* in the figure, and if no precautions are taken the display will contain domains with each of these profiles. The optical transmission of obliquely passing light is different in each case, and so the display has a patchy appearance. Moreover, the domains are separated by a transition line that is clearly visible between polarizers and deteriorates the image.

Parts *c* and *d* of the figure show that the occurrence of two possible orientations can be avoided by giving the director at each substrate a bias tilt. So an alignment method for liquid crystals should not only provide a preferential direction for the molecules in the plane of the substrate, it should also define a suitable surface bias tilt, typically between 2° and 10°.

The old technique³ of rubbed polymer films has been brought to high enough sophistication to meet these requirements⁴. The selection and processing of these polymers is partly a science and partly a craft, or even an art. But it generates dust, which conflicts with the extremely clean manufacturing conditions needed, and there is a risk of electrostatic damage to the thin film transistors in the displays commonly used for television and computer monitors.

In 1992, researchers at Hoffman-La Roche in Basel and NIOPIK in Moscow achieved in-plane control of liquid-crystal orientation, though without bias tilt, using linearly polarized ultraviolet light⁵. The light breaks bonds that happen to be oriented parallel to its polarization direction, and then new bonds form perpendicular to this direction.

A lot of work has been done since 1992 to qualify this photoalignment technique for application in the liquid-crystal display industry, but a satisfactory value for the bias tilt has not been reported. Attempts to break the symmetry of the liquid-crystal layer on top of the polymer film by using obliquely incident light were without success, because the liquid crystal orients itself parallel to the molecules of the substrate, that is, perpendicular to the polarization direction of the ultraviolet light.

The paper of Schadt and colleagues¹

reports on a material that, instead, aligns the liquid crystal more or less parallel to the polarization direction of the light. This is crucial for making photoalignment a viable alternative to the conventional rubbing technique. There are still practical issues to be addressed, however, such as the processing of the materials, their stability and their transparency, and the contamination of the liquid crystal by the orientation layer or its solvent.

The photoalignment technique can not only simplify display manufacture. It can also be used to make novel, better displays by forming patterns of orientation within each display element^{1,6} to reduce the dependence of visibility on the direction of view — an obvious shortcoming of liquid-crystal television and computer screens.

Several approaches to this problem are now being industrialized. They include adding birefringent compensators, alternative liquid-crystal configurations in which twist angle varies in response to electric fields, combinations of collimating and diffusing foils that enclose the liquid-crystal cell, and the division of picture elements into differently oriented subpixels or subpixels in series with small capacitors. It is an open question which approach will become the mainstream technology for future high-quality liquid-crystal displays. The results of Schadt and

colleagues certainly increase the chances of the subpixel method.

One might pursue this topic to replace existing techniques, to improve existing products or to make entirely new components or devices. For example, the new technique could be used to make stacks of birefringent layers with, in principle, independent control over the orientation of each layer. Among other things, this could be used as a 'copy-proof' way of making images that are visible in polarized light only⁷. The paper of Schadt, Seiberle and Schuster adds an intriguing item to the list of exciting things that can be done at the interface of optics, polymer chemistry and liquid-crystal device technology. □

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- Schadt, M., Seiberle, H. & Schuster, A. *Nature* **381**, 212–215 (1996).
- Schadt, M. & Helfrich, W. *Appl. Phys. Lett.* **18**, 127–128 (1971).
- Zocher, H. *Naturwissenschaften* **13**, 1015–1021 (1925).
- van Aerle, N. J. A. M. *J. Soc. Inf. Display* **2**, 41–46 (1994).
- Schadt, M., Schmitt, K., Kozinkov, V. & Chigrinov, V. *Jap. J. appl. Phys.* **31**, 2155–2164 (1992).
- Iimura, Y., Saitoh, T., Kobayashi, S. & Hashimoto, T. *J. Photopolym. Sci. Technol.* **8**, 257–262 (1995).
- Schadt, M., Seiberle, H., Schuster, A. & Kelly, S. M. *Jap. J. appl. Phys.* **34**, L764–L767 (1995).

PROTEIN TARGETING

The ribosome talks back

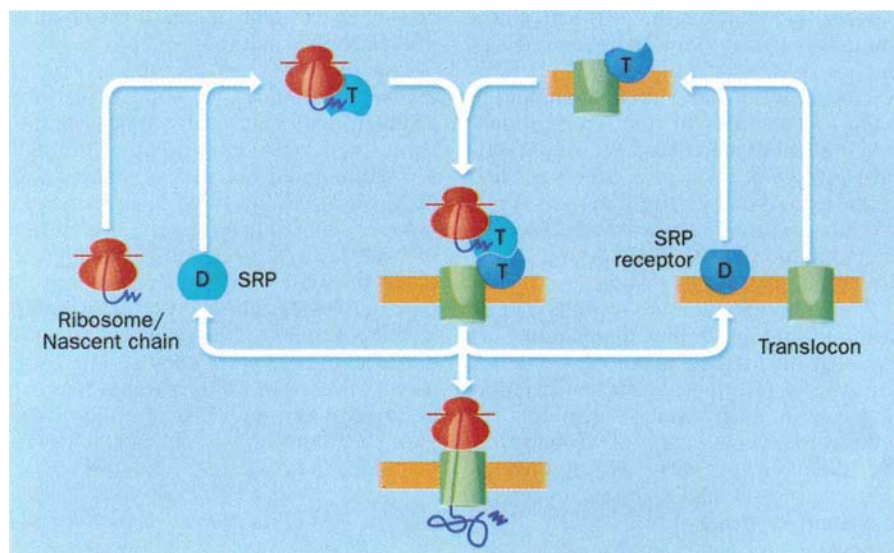
Ted Powers and Peter Walter

THE synthesis of most mammalian membrane and secretory proteins begins on free, cytosolic ribosomes that are targeted co-translationally to the membrane of the endoplasmic reticulum (ER). The mechanism by which these nascent proteins are selected requires three distinct, directly interacting GTPases which serve as adapters between the translation apparatus in the cytosol and the protein translocation apparatus, the translocon, in the ER membrane¹. The function and regulation of these GTPases has become the object of intensive study, and now, as described on page 248 of this issue², Bacher *et al.* have made the intriguing observation that the GTP-binding activity of one of these GTPases is modulated by the ribosome itself.

To place this work in context, proteins are synthesized by a common pool of ribosomes, but only those proteins that carry a hydrophobic signal peptide are imported into the ER. These include all transmembrane proteins, most secreted proteins and most proteins destined to be stored in the lumen of intracellular organelles such as the Golgi apparatus,

lysosomes and endosomes. As the protein is synthesized, the signal-recognition particle (SRP) binds to both the hydrophobic signal peptide and to the ribosome, directing them to the SRP receptor on the cytosolic surface of the ER membrane. After the complex has docked, the protein is translocated across the ER membrane through the translocon, and may remain in the ER lumen or be distributed to other destinations in the cell.

The component in question here is the 54K subunit (SRP54) of the signal-recognition particle, which is the cytosolic ribonucleoprotein that first identifies those ribosomes that synthesize nascent chains bearing ER-directed signal sequences¹. SRP is composed of an RNA and six polypeptides, of which SRP54 can be viewed as the most central player. It contains an amino-terminal GTPase domain and a carboxy-terminal domain which binds directly to signal sequences and SRP RNA. Homologues of SRP54, as well as the small motif in SRP RNA to which it binds, can be traced all the way to eubacteria and archaeobacteria, where they are



Model for regulation of GTP binding to the signal-recognition particle (SRP) and its receptor during co-translational targeting of pre-proteins to the membrane of the endoplasmic reticulum (ER). The branch on the left depicts binding of SRP to a ribosome-nascent polypeptide that contains a signal sequence. As shown by Bacher *et al.*², the ribosome acts as a GTP loading factor for SRP54 and locks it into its GTP-bound form (indicated as 'T'), thus 'turning on' SRP for docking to its receptor at the ER membrane. The branch on the right is speculative and proposes that a component of the translocon similarly turns on the SRP receptor by stabilizing the GTP-bound form of the α -subunit of the SRP receptor; the role of the β -subunit of the SRP receptor in targeting is unclear, although a functional GTP-binding domain is required for translocation (S. Ogg and P. Walter, unpublished data). Binding of GTP to SRP54 and the α -subunit of the SRP receptor is required for their tight association and leads to formation of the ribosome-membrane junction. Subsequent hydrolysis of GTP to GDP (indicated as 'D') by SRP and its receptor results in their dissociation and allows for another round of targeting.

likely to perform similar functions in protein targeting.

After binding to ribosomes and signal sequences, SRP interacts with its receptor, an ER membrane protein composed of two subunits, SR α and SR β , both of which also contain GTPase domains. In the presence of GTP, the SRP receptor causes SRP to be released from the ribosome-nascent chain, and the latter becomes tightly associated with components of the translocon, composed principally of the heterotrimeric Sec61p complex³. These targeting events only require GTP binding, not its hydrolysis, as non-hydrolysable analogues can substitute and promote a round of targeting. GTP hydrolysis is required for a subsequent step that releases SRP from the receptor so that it can return to the cytosol⁴. But how, mechanistically, do the three individual GTPases — SRP54, SR α and SR β — collaborate to drive a targeting cycle?

The first insight into this question came by studying the interaction between purified SRP and its SRP receptor *in vitro*⁵. Monitoring GTP and GDP binding to individual proteins by ultraviolet crosslinking, it was found that SRP receptor both promoted GTP binding to SRP54 and then activated its GTPase. Interestingly, SRP receptor did not affect the affinity of SRP54 for GDP, in contrast to other, well-characterized guanine nucleotide exchange factors. This suggested that SRP receptor acts as a novel 'GTP-loading fac-

tor', increasing the affinity of SRP54 for GTP directly. Furthermore, nucleotide binding to SRP54 was blocked in the presence of synthetic signal peptides. By extrapolation, these results pointed to a model whereby SRP is stabilized in a nucleotide-free state by signal sequences within a targeting complex; subsequent interaction with the receptor at the membrane facilitates release of the signal sequence in exchange for binding of GTP to SRP.

Enter Bacher *et al.*², who have now used similar assays to include bona fide ribosome-nascent chain complexes. Their most important observation is that the affinity of SRP54 for GTP is increased by about an order of magnitude in the presence of a ribosome that carries a nascent chain with a signal sequence. Thus before encountering the SRP receptor, the ribosome already acts as a GTP-loading factor for SRP54, thereby moving the step in which GTP enters the targeting cycle up by one notch. The GTP-loading activity of the ribosome apparently overrides the inhibitory effect caused by signal peptides alone.

These results are appealing because they offer a simple mechanism for how targeting is regulated. In the presence of a signal sequence, SRP binds tightly to the ribosome and becomes 'turned on', by GTP binding, for docking to its receptor. The SRP receptor would then stabilize SRP54 in its GTP-bound state and induce

dissociation of SRP from the ribosome. In turn, this would lead to release of the signal sequence by SRP54 and docking of the ribosome-nascent chain at the translocon. Finally, GTP hydrolysis would allow dissociation of SRP from the membrane.

Bacher *et al.* also observe that the GTP-loading activity of the ribosome affects even free SRP54 (that is, in the absence of SRP RNA and the other SRP proteins), indicating that SRP54 interacts directly with some ribosomal component or components. It will be interesting to ask in future experiments whether this activity is caused by the same ribosomal component(s) involved in interactions with other well-characterized GTPases that bind to the ribosome, such as elongation factors.

Intriguingly, the GTPase domains of the SR α subunit of the SRP receptor and of SRP54 are phylogenetically related, being more similar to each other than to any other member of the superfamily of GTPases. As for SRP54, SR α must also be in its GTP-bound form to engage SRP⁶, and their prokaryotic homologues engage in a similarly symmetrical interaction. Bacterial SRP54 and SR α function as reciprocal GTPase-activating proteins for each other, each needing to be bound to GTP to show this activity⁷. It is therefore likely that SR α will similarly be turned on by interacting with another component that promotes its GTP binding. A likely candidate would be the translocon itself.

According to this view, the GTPase cycles of SRP and the SRP receptor would be coupled directly to their roles as molecular matchmakers, recruiting cytosolic and membrane-bound components, respectively, to catalyse formation of the ribosome-membrane junction (see figure). Each would be turned on and ready to interact after having found its appropriate cargo. Although this model incorporates the requirement for two GTPase switches, the need for a third, in SR β , remains a mystery — indicating that, even with these new insights and speculations, we are still far from a full picture of events. □

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1. Walter, P. & Johnson, A. E. *Rev. Cell Biol.* **10**, 87–119 (1994).
2. Bacher, G., Lütke, H., Jungnickel, B., Rapoport, T. A. & Dobberstein, B. *Nature* **381**, 248–251 (1996).
3. Görlich, D. & Rapoport, T. A. *Cell* **75**, 615–630 (1993).
4. Connolly, T., Rapiejko, P. J. & Gilmore, R. *Science* **252**, 1171–1173 (1991).
5. Miller, J. D., Wilhelm, H., Gierasch, L., Gilmore, R. & Walter, P. *Nature* **366**, 351–354 (1993).
6. Rapiejko, P. J. & Gilmore, R. *J. Cell Biol.* **117**, 493–503 (1992).
7. Powers, T. & Walter, P. *Science* **269**, 1422–1424 (1995).

Deuteronomy and numbers

David N. Schramm and Michael S. Turner

FOUR light isotopes — deuterium, ^3He , ^4He and ^7Li — were produced by nuclear reactions a few seconds after the Big Bang. New measurements of deuterium in high-redshift hydrogen clouds by Tytler *et al.* (page 207 of this issue¹) and of ^3He in the local interstellar medium by Gloeckler and Geiss (page 210²) provide further confirmation of Big Bang nucleosynthesis and new insight into the density of ordinary 'baryonic' matter.

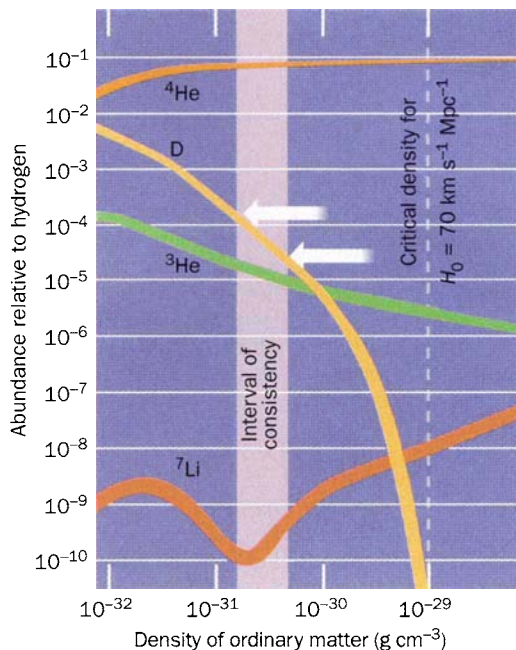
The large primeval abundance of ^4He , between 22 and 25 per cent of all the elements by mass, provided the first confirmation of Big Bang nucleosynthesis. The other light elements are produced in far smaller amounts but play important roles in testing this theory because their yields are much more sensitive to the density of matter than ^4He is. Measuring them provides the easiest test of the Big Bang theory and the best determination of the mean density of ordinary matter in the Universe³ (see figure). The theory of nucleosynthesis can account for the primeval abundances of all four light elements, provided the present-day baryon density is between 1.5 and $4.5 \times 10^{-31} \text{ g cm}^{-3}$.

Deuterium is the best baryometer⁴ because its production in the Big Bang decreases rapidly with density and its later chemical evolution is simple — stars only destroy it. (The term 'chemical evolution' actually refers to nuclear processing.) In the 1970s the first measurements of the deuterium abundance in the Solar System and in the local interstellar medium indicated that matter in the form of baryons could account for at most 15 per cent of the critical density needed to 'close' the Universe. That conclusion still holds. Because of possible depletion in stars, the present-day deuterium abundance is a lower limit on the primeval value and thereby an upper limit to the baryon density.

But there is a way of measuring the deuterium abundance in very primitive samples of the cosmos where stellar depletion would not be a concern⁵. The deuterium Lyman- α line, just $0.33 \text{ \AA}$ blueward of the equivalent line in hydrogen, could be detected in high-redshift hydrogen clouds backlit by distant quasars. From such a measurement the baryon density could be inferred to an accuracy of 15 per cent or so.

For very distant clouds, these ultraviolet lines are shifted into the visible spec-

trum, where ground-based instruments can detect them. But it is no mean feat. Isolated clouds of high column density and low velocity dispersion must be found, and a very high spectral resolution and signal-to-noise ratio is needed. Only now, with the 10-metre Keck Telescope and its high-resolution echelle spectrograph (HIRES) is the dream becoming reality. In the past eighteen months three detec-



Summary of Big Bang production of the light elements. The widths of the curves indicate 2σ theoretical uncertainties, and the vertical band is the consistency interval where the abundances of all four light elements agree with their measured primeval abundances. The critical density, which depends on the square of the Hubble constant, is indicated for $H_0 = 70 \text{ km s}^{-1} \text{ Mpc}^{-1}$. The arrows indicate the two conflicting new deuterium abundances measured by Songaila *et al.*⁶ (upper) and Tytler *et al.*¹ (lower). (Figure courtesy of C. Copi.)

tions and four tentative detections have been reported. The clouds studied are old, with redshifts between 2.6 and 4.7, and pristine, with heavy-element abundances all below 10^{-2} of that in the Solar System (heavy elements are made by stellar processes, not in the Big Bang).

The two clouds studied by Tytler *et al.*¹ have deuterium abundances relative to hydrogen of (2.3 ± 0.3) and $(2.5 \pm 0.3) \times 10^{-5}$, implying a baryon density of $(4.4 \pm 0.6) \times 10^{-31} \text{ g cm}^{-3}$. The other firm detection^{6,7} has a much higher abundance, $(2.0 \pm 0.4) \times 10^{-4}$, and indicates a baryon density of $(1.3 \pm 0.3) \times 10^{-31} \text{ g cm}^{-3}$. The four tentative detections fall in between.

Any detection is most conservatively interpreted as an upper limit to the primeval abundance, because of the possi-

bility of a low-column-density hydrogen cloud along the line of sight but with a slightly different redshift, fortuitously located to mimic the deuterium feature (an interloper). The high-abundance measurement might be due to a hydrogen interloper, with the low value being the true primeval abundance. In that case, the baryon density is at the high end of the range anticipated, around 5 per cent of the critical density (assuming a Hubble constant of $70 \text{ km s}^{-1} \text{ Mpc}^{-1}$).

Rugers and Hogan⁸ estimate the chance probability of an interloper at less than 10 per cent, however, and suggest a more radical hypothesis. They propose that in the clouds studied by Tytler *et al.*, stellar processing has reduced the deuterium abundance by a factor of ten. The abundance of heavy elements suggests that little stellar processing has occurred, but it may be that the cloud is very inhomogeneous and the heavy elements have been dispersed. This is not implausible, because the material giving rise to the absorption lines has only a tiny fraction of the cloud's mass.

Should the primeval deuterium abundance be so high, the inferred baryon density is very low (around 1 per cent of critical density for a Hubble constant of $70 \text{ km s}^{-1} \text{ Mpc}^{-1}$) and the case for exotic, nonbaryonic dark matter becomes iron-clad — all estimates for the total matter density exceed 10 per cent, and many do so by a wide margin. But a high primeval deuterium abundance implies that more than 90 per cent of the material in the local interstellar medium has been through stars at least once, because the deuterium abundance there is $(1.6 \pm 0.1) \times 10^{-5}$ (ref. 9). This contradicts many models of the chemical evolution of our Galaxy.

Further doubt is cast on a high abundance by the fact that, in stars, deuterium is burnt to ^3He . According to conventional stellar models, that is much more difficult to destroy, so a high primeval deuterium abundance should produce a high ^3He abundance. But Gloeckler and Geiss² find that the local ^3He abundance is $(2.1 + 0.9/-0.8) \times 10^{-5}$, precluding this possibility.

Conventional stellar wisdom also predicts that the sum of deuterium and ^3He

1. Tytler, D., Fan, X.-M. & Burles, S. *Nature* **381**, 207–209 (1996).
2. Gloeckler, G. & Geiss, J. *Nature* **381**, 210–212 (1996).
3. Copi, B. J., Schramm, D. N. & Turner, M. S. *Science* **267**, 192–199 (1995).
4. Epstein, R. I., Lattimer, J. M. & Schramm, D. N. *Nature* **263**, 198–202 (1976).
5. Adams, F. T. *Astr. Astrophys.* **50**, 461–462 (1976).
6. Songaila, A., Cowie, L. L., Hogan, C. J. & Rugers, M. *Nature* **368**, 599–603 (1994).
7. Carswell, R. F., Rauch, M., Weymann, R. J., Cooke, A. J. & Webb, J. K. *Mon. Not. R. astr. Soc.* **268**, L1–L4 (1994).
8. Rugers, M. & Hogan, C. J. *Astrophys. J.* **459**, L1–L4 (1996).
9. Linsky, J. L. *et al. Astrophys. J.* **451**, 335–351 (1995).
10. Iben, I. & Turan, J. W. *Astrophys. J.* **220**, 980–995 (1978).
11. Jungman, G., Kamionkowski, M., Kosowsky, A. & Spergel, D. *Preprint astro-ph 9512139*.

should grow, due to ^3He production from hydrogen by low-mass stars¹⁰. The measurement by Gloeckler and Geiss is inconsistent with this prediction because the sum of the local deuterium and ^3He abundances is essentially equal to that inferred for the Solar System 4.5 billion years ago. At the moment, any argument for or against a particular value of the primeval deuterium abundance based upon the chemical evolution of ^3He has little weight.

There are also pieces missing from the ^7Li story. Its abundance in the atmos-

pheres of the oldest stars in the halo of the Galaxy is $(1.4 \pm 0.3) \times 10^{-10}$. If this is the primordial value, it favours a low baryon density. However, some stellar models indicate that these stars could have reduced their initial ^7Li abundance by a factor as large as two, which would be consistent with a high baryon density.

It is a good bet that within a few years the primeval deuterium abundance will be determined unambiguously, and with it the value of the baryon density. An important check may come a few years later from detailed mapping of the anisotropy

of the cosmic background radiation, which provides an independent way of determining the baryon density to similar precision¹¹. Once the baryon density is known, nuclear physics in the early Universe can teach us about the 'chemistry' of ^3He and ^7Li in the contemporary Universe. □

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HUMAN GENETICS

Blindness and the X

Sean E. Egan and Roderick R. McInnes

POSITIONAL cloners, explorers of undefined but promising genetic territory, are as vulnerable to confounding data as were any of their nineteenth-century counterparts in mapping the head of the Nile. But the longest lasting chromosomal walk to date has now ended happily, 12 years after Bhattacharya and his colleagues¹ found a locus for X-linked retinitis pigmentosa (xLRP) on the short arm of the X chromosome.

As reported in the May issue of *Nature Genetics*², two previously competing groups joined in an admirable collaboration to find an RP gene on Xp at the locus (*RP3*) associated with more cases of RP than any other known, up to around 20% in some populations. Discovery of the new gene, named *RPGR* (for retinitis pigmen-

tosa GTPase regulator) has important implications for retinal biology, and for patients with the disease and their families.

Retinitis pigmentosa is a relatively common genetic disorder which affects 1 in 4,000 people and is caused by the premature death of photoreceptors. The result is progressive night blindness, contraction of the visual field and, finally, complete loss of sight. X-linked RP is one of the more severe forms; it usually becomes manifest within the first two decades of life, and progresses to blindness within 10–20 years³.

What made the search for *RP3* so tortuous? First, the genetic analysis of xLRP was plagued by unexpected locus heterogeneity. The initial impression of a single xLRP locus¹ has been expanded to include

four putative loci (*RP2*, *RP3*, *RP6* and *RP15*) between Xp11.22 and Xp22.13 (refs 4–8), although the genetic evidence is undeniable only for the existence of *RP2* (in Xp11.3–11.23) and *RP3* (in Xp21.1)^{5,6}—see figure. Ironically, the probe, L1.28, that was thought to be revealing a single locus in the 1984 work was demonstrating linkage to both *RP2* and *RP3*, because the DNA sequence it detects, DXS7, is situated between these two loci^{4,5} (see figure).

Second, whereas large chromosomal deletions are generally invaluable guides to disease genes, a large deletion in one RP patient (called B.B.) may have misdirected the search. *RP3* mapped close to the B.B. deletion and was initially presumed to be within it. But studies of other RP patients with deletions in the region², and additional mapping⁹, indicated that *RPGR* is about 400 kilobases centromeric to the nearer edge of the B.B. deletion. In the end, the good reputation of deletions in positional cloning was restored by a

Retinitis pigmentosa: the Ran connection

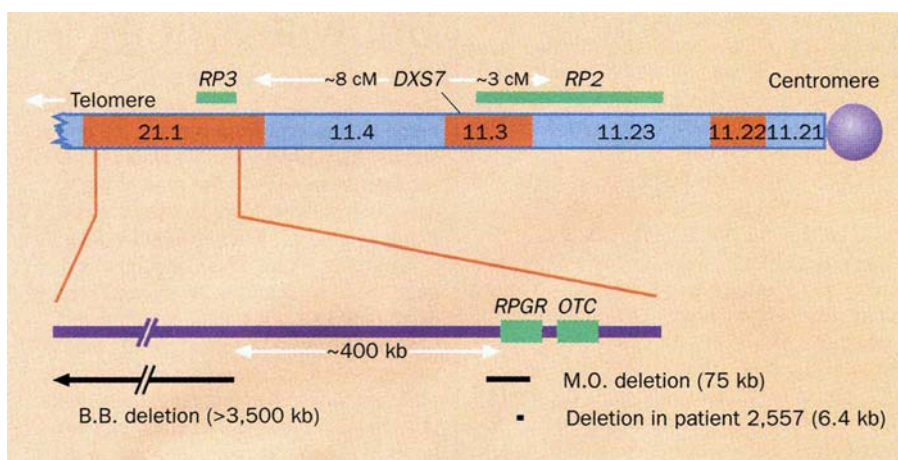
WHAT biological riches might lie in the *RPGR* coding sequences that Meindl *et al.*² have unearthed? The predicted 90K *RPGR* protein bears significant homology to the RCC1 family of guanine nucleotide exchange factors. RCC1 activates the replacement of guanosine diphosphate (GDP) by guanosine triphosphate (GTP) on the small GTPase, Ran (ref. 13). The RCC1–Ran complex regulates the transport of two different sets of macromolecules through the nuclear pore complex (NPC)¹⁴. RNA is shuttled out of the nucleus when RCC1, a nuclear protein, activates GTP loading of nuclear Ran. On the cytoplasmic side of the NPC, Ran–GDP first initiates the association of a large complex composed of the NPC, nuclear-targeted proteins and a multisubunit transporter. Once this complex is formed, the nuclear-targeted proteins are shuttled through the pore when the

Ran-bound GDP is exchanged for GTP (ref. 15). So Ran–GDP and Ran–GTP each play distinct roles on both sides of the nuclear membrane in mediating the assembly and dissociation of multi-component complexes.

It seems safe to suggest that the *RPGR* protein may also regulate nucleotide exchange on Ran, or on a Ran-like protein. Unlike RCC1, however, *RPGR* contains a long, charged carboxy-terminal domain which ends with a CAAX box, probably targeting this protein for geranylgeranylation and insertion into a cellular membrane. In addition, *RPGR* does not contain amino-terminal nuclear localization sequences and may therefore reside in some other subcellular compartment. These characteristics of *RPGR*, and the presence of other novel RCC1-like proteins in the public databases², suggest that Ran proteins may regulate many cellular functions and that different ex-

changers will activate GTP-loading of Ran at different cellular locales. Finding out what the *RPGR* protein does will require identifying its subcellular location, its target GTPase and the macromolecular company it keeps.

The answers to these basic biochemical questions should explain the pathogenesis of this type of xLRP, particularly the unique sensitivity of the retina to mutations in *RPGR*—an important issue, because the retina and retinal pigment epithelium have the lowest levels of *RPGR* expression, by far, of all tissues examined². Perhaps the *RPGR* protein has a preferred retinal partner, such as a Ran-like protein that is not activated by other RCC1 proteins in the retina. In addition, phenotypic questions such as whether ciliary abnormalities occur in xLRP (ref. 16), can now be subjected to molecular scrutiny, and may point to other tissues in which *RPGR* is essential. S. E. E. & R. R. Mcl.



Map of part of the short arm of human chromosome X (Xp). It depicts the relationships between the *RP2* and *RP3* loci; the distance of the newly identified retinitis pigmentosa GTPase regulator (*RPGR*) gene from the deletion in patient B.B.; and the deletion in patient M.O. (ref. 2) that was critical in identifying *RPGR*. The small *RPGR* deletion independently found by Roepman *et al.*¹⁰ in patient 2,557 is also shown. OTC, ornithine transcarbamylase, a well-known locus on Xp; cM, centimorgan. (Figure adapted from ref. 9.)

deletion identified in RP patient M.O., who lacked a 75-kilobase DNA fragment within which Meindl *et al.*² ultimately found *RPGR*.

The *RP3* locus is closely linked to the disease gene in 70–90% of xLRP patients, depending on the population being studied. Consequently, Meindl *et al.* expected that mutations in *RPGR* would exist in most xLRP patients. Although this may still prove to be the case, the authors found abnormalities in the gene in only 7 of 74 xLRP patients examined. Another xLRP patient with a small deletion in *RPGR* has also been reported¹⁰. Meindl *et al.* suggest that trivial explanations, such as unidentified *RPGR* exons or undetected mutations, may account for the low mutation frequency they observed. Alternatively, they acknowledge that *RPGR* may not be the only RP gene embraced by the *RP3* locus in Xp21.1. Whatever the explanation, the final accounting of RP genes on Xp21.1 must explain the presence of disease in patient B.B.. The

massive B.B. deletion of at least 3.5 million base pairs may include another RP locus, or an *RPGR* regulatory element situated at an unprecedented distance from the gene.

The discovery of *RPGR* will do much to facilitate identification of the gene re-

CARBON CYCLE

A quickening on the uptake?

Michael Bender

WHERE does the carbon dioxide that our industrial society pumps into the atmosphere go? This basic question, vital for an understanding of the most important of environmental problems, is still not answered with precision or with absolute confidence. On page 218 of this issue¹ Keeling *et al.* show that, between 1991 and 1994, much of the carbon was trapped in the growing forests of the Northern Hemisphere. But of course there is more to the story than that.

This paper is the second landmark contribution from Ralph Keeling and collaborators on the distribution of oxygen (O_2) concentration in air and its implications for the fate of CO_2 added to the atmosphere by fossil fuel burning and changes in land use. The authors extend earlier studies^{2,3} by providing multi-year records of how the O_2/N_2 ratio varies in both hemispheres. They show that the O_2 content of air decreased between 1991 and 1994, but more slowly than is implied by the rate of fossil fuel combustion (which takes oxygen to form CO_2 and water). The difference suggests the existence of a large oxygen source, which can only be from photosynthesis associated with the net growth of the land biosphere.

Of a total anthropogenic CO_2 produc-

sible for RP in many patients. Up to 40% of patients do not have a family history to suggest whether their RP is autosomal or X-linked³. This uncertainty will now be resolved for male patients with *RPGR* mutations. Furthermore, women in RP families segregating *RPGR* alleles can now be tested to see whether they are carriers. Mutation analysis of the gene will also reveal whether families with other retinal disorders (including a form of congenital stationary night blindness (CSNB3)¹¹ and an X-linked cone dystrophy¹²), represent distinct loci or are simply *RPGR* alleles.

In a News and Views article on the 1984 xLRP paper¹, Miranda Robertson prophesied that "Progress... will require patience and ingenuity". It did, but the discovery of *RPGR* has now brought a full understanding of RP closer to realization. □

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tion of about 6 Gt C yr⁻¹ (gigatonnes of carbon per year), the authors calculate that the global land biosphere sequestered 2.0 ± 0.9 Gt C yr⁻¹ over the 4 years of their study. The atmospheric CO_2 content increased by 2.3 Gt C yr⁻¹, so ocean uptake must have been 1.7 ± 0.9 Gt C yr⁻¹ on average. Keeling *et al.* go on to show that the O_2/N_2 ratio is lower in the Northern Hemisphere, as expected from the fact that most fossil fuel is burned there. But the interhemispheric O_2/N_2 gradient is smaller than one would expect if the land biosphere sequestered CO_2 uniformly over Earth's continental surface. It seems that the land biosphere of the Northern Hemisphere dominates this process.

The three key flux terms calculated by Keeling *et al.* — net CO_2 uptake by the global land biosphere, the land biosphere of the Northern Hemisphere and the oceans — are all in good agreement with estimates from independent 'tracer' methods, in which fluxes are calculated from the distribution and isotopic composition of CO_2 in air and sea water. Each method requires somewhat unsatisfying assumptions and has different sources of error. Convergence of various estimates gives us confidence that we are correctly

1. Bhattacharya, S. S. *et al.* *Nature* **309**, 253–255 (1984).
2. Meindl, A. *et al.* *Nature Genet.* **13**, 35–42 (1996).
3. Heckenlively, J. R. *Retinitis Pigmentosa* (Lippincott, Philadelphia, 1988).
4. Musarella, M. A. *et al.* *Genomics* **8**, 286–296 (1990).
5. Ott, J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **87**, 701–704 (1990).
6. Rosenfeld, P. J., McKusick, V. A., Amberger, J. S. & Dryja, T. P. *J. Med. Genet.* **31**, 903–915 (1994).
7. McGuire, R. E. *et al.* *Am. J. hum. Genet.* **57**, 87–94 (1995).
8. Aldred, M. A. *et al.* *J. Med. Genet.* **31**, 848–852 (1994).
9. Fujita, R. *et al.* *Am. J. hum. Genet.* (in the press).
10. Roepman, R. *et al.* *Hum. molec. Genet.* (in the press).
11. Bergen, A. A. B., ten Brink, J. B., Riemsdijk, F., Schuurman, E. J. M. & Tijmes, N. *Hum. molec. Genet.* **4**, 931–935 (1995).
12. Bergen, A. A. B. *et al.* *Genomics* **18**, 463–464 (1993).
13. Rush, M. G., Drivas, G. & D'Eustachio, P. *BioEssays* **18**, 103–112 (1996).
14. Görlich, D. & Mattaj, J. W. *Science* **271**, 1513–1518 (1996).
15. Nehrbass, U. & Blobel, G. *Science* **272**, 120–122 (1996).
16. Barrong, S. D. *et al.* *Arch. Ophthalmol.* **110**, 706–710 (1992).

deducing evolving carbon fluxes during the past 15 years.

Three independent tracer approaches constrain rates of CO_2 uptake by the oceans. One approach is based on the uptake rate by the oceans of $^{14}\text{CO}_2$ generated by nuclear bomb tests, on the difference in partial pressures of CO_2 between the atmosphere^{4,5} and the surface ocean, and models of ocean mixing⁶.

A second method examines the rate of change of the stable-isotope ratio of carbon in the atmosphere and sea water⁷⁻⁹. Burning of fossil fuel CO_2 (with $^{13}\text{C}/^{12}\text{C} \sim -28$ parts per thousand, relative to the PDB-1 standard) and its invasion into the oceans is causing the isotope ratio of total dissolved CO_2 in surface sea water ($^{13}\text{C}/^{12}\text{C} \sim +2$ parts per thousand) to decrease.

The third technique¹⁰ is to measure the CO_2 concentration and the $^{13}\text{C}/^{12}\text{C}$ ratio of atmospheric CO_2 as a function of latitude, in order to partition the total uptake rate between the oceans and the land biosphere (CO_2 uptake by the land biosphere discriminates against the heavier isotope). All three CO_2 tracer approaches estimate oceanic uptake at about 2 Gt C yr^{-1} for various periods between 1980 and 1994.

On land, recent history is more complicated. The global net CO_2 uptake by the land biosphere estimated by Keeling *et al.*¹, 2.0 Gt C yr^{-1} for 1991–94, is in good agreement with that of the last tracer method¹⁰, 2.6 Gt C yr^{-1} for 1992–93, and both these approaches indicate that the land biosphere was an important CO_2 sink during the early years of the current decade. But in contrast, several studies¹¹ suggest that between 1980 and 1989 the biosphere was roughly in balance.

The CO_2 mass balance during this earlier period is constrained by the modelled estimates of oceanic CO_2 uptake outlined above, as well as studies of the O_2/N_2 ratio of air in the firm (the partly compacted layer of snow) at the surface of polar glaciers¹². According to these results, fossil fuel emissions during the 1980s ($\sim 5.4 \text{ Gt C yr}^{-1}$) were balanced by the atmospheric increase (3.2 Gt C yr^{-1}) and oceanic uptake ($\sim 2 \text{ Gt C yr}^{-1}$). By the 1991–94 period, emissions had risen to about

6 Gt C yr^{-1} , ocean uptake remained at about 2 Gt C yr^{-1} , and the atmospheric increase fell from ~ 3 to $\sim 2 \text{ Gt C yr}^{-1}$. Tracer studies summarized above indicate that the global land biosphere took up the imbalance, $\sim 2 \text{ Gt C yr}^{-1}$.

While tracer studies suggest that the global land biosphere was nearly in CO_2 balance during the 1980s, they nevertheless indicate that some areas were a net sink. A largely equatorial CO_2 source from deforestation was compensated for by sequestration of CO_2 elsewhere. If deforestation has added 1.6 Gt C yr^{-1} of CO_2 since 1980 (ref. 13), then the rest of the land biosphere took up about 1.6 Gt C yr^{-1} during the decade of the 1980s and 3.6 Gt C yr^{-1} between 1991 and 1994. Carbon-isotope tracer modelling¹⁰ agrees with the O_2/N_2 measurements of Keeling *et al.* in indicating net CO_2 uptake of $\sim 3 \text{ Gt C yr}^{-1}$ by the land biosphere above 30°N , and measurements of the CO_2 uptake rate in a northern temperate forest are consistent with such rapid uptake¹⁴. Plausible causes include CO_2 fertilization of the land biosphere^{15,16}, nitrogen fertilization resulting from industrial emissions¹⁷, and warmer temperatures and increased precipitation¹⁸ (but see ref. 19 for a contrary view).

During the past year or so, the rate of atmospheric CO_2 rise has accelerated and is once again comparable to the average rate during the 1980s (P. Tans, personal communication). This observation emphasizes the dynamic nature of the carbon cycle. Although ocean CO_2 uptake does fluctuate, the land biosphere is probably the main cause of variability, some of which appears to be caused by El Niño events⁴. More generally, land biosphere variability must reflect changes in the small imbalance between photosynthesis and respiration, with their gross fluxes of about 100 Gt C yr^{-1} . Variability in that imbalance presumably relates to changes in climate, but the nature of the link is unknown. □

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DAEDALUS

Snow growing

GLOBAL warming poses several kinds of threat. Perhaps the most ominous is the rising of sea level from melting ice and snow. Much of the world's water is held as 'eternal snows' on the tops of high mountain ranges. If those snows stop being so eternal, there will be trouble.

A mountain top, says Daedalus, is a good place to store frozen water. The cold of altitude keeps it below zero; snow precipitates on it from the clouds that sweep over it, and piles up to even higher altitudes where the temperature is colder still. However, the stored snow has two ways of escape. It can evaporate in the sunlight, and it can descend under gravity — either rapidly as an avalanche, or slowly and viscously as a glacier. Daedalus now plans to frustrate both modes of escape.

During the Second World War, there was a scheme to adapt a North Atlantic iceberg as an aircraft carrier. Natural ice is rather weak; so the boffins planned to freeze their own iceberg, from water with a little wood-pulp in it. The resulting fibre-reinforced ice was admirably tough, and also melted much more slowly. Innumerable fibres protruded from the melting surface, forming a blanket which impeded heat transfer.

So, says Daedalus, what we need is some way of launching fine fibres into the wind traversing the Andes, the Himalayas and the other snowy mountain ranges. Once their snow is reinforced with fine fibres, it will be much more eternal. It won't creep like a glacier, nor tumble like an avalanche; and its insulated, whiskery surface will evaporate far more slowly. Less than one per cent of fibre has a dramatic effect on the toughness of ice or snow. Even so, millions of tons of fibre will be needed.

Daedalus's bold idea is to make it from our growing mountain of plastic waste. Long-chain polymers easily form fibres; some process of air-blown melt-spinning or violent pulverization should give a suitably fine, feathery, fibrous dust. Released into the air upwind of the mountains, it will be incorporated into the snowfall at the summit. But volcanic dusts are often blown halfway round the world on the wind. So rather than transporting the powder at great expense to the mountainous regions, Daedalus hopes to release it at the site of manufacture. Modern meteorology may be able to identify just that favourable wind which will carry it from the fibre factory to the far mountain of its destiny. The world's plastic waste will be put to good use, the sea level will stop rising, and the high eternal snow will rise instead. Mountaineers will tread more surely on its toughened surface. David Jones

- Keeling, R. F., Piper, S. C. & Heimann, M. *Nature* **381**, 218–221 (1996).
- Keeling, R. F. & Shertz, S. R. *Nature* **358**, 723–727 (1992).
- Bender, M., Ellis, T., Tans, P., Francey, R. & Lowe, D. *Globi biogeochem. Cycles* **10**, 9–21 (1996).
- Keeling, C. D. *et al.* in *Aspects of Climate Variability in the Pacific and Western Americas* (ed. Peterson, D. H.) 165–236 (American Geophysical Union, Washington DC, 1989).
- Conway, T. J. *et al.* *J. geophys. Res.* **99**, 22831–22855 (1994).
- Sarmiento, J. L., Orr, J. C. & Siegenthaler, U. *J. geophys. Res.* **97**, 3621–3645 (1992).
- Quay, P. D., Tilbrook, B. & Wong, C. S. *Science* **256**, 74–79 (1992).
- Tans, P. P., Berry, J. A. & Keeling, R. F. *Globi biogeochem. Cycles* **7**, 353–368 (1993).
- Heimann, M. & Maier-Reimer, E. *Globi biogeochem. Cycles* **10**, 89–110 (1996).

- Ciais, P., Tans, P. P., Trolier, M., White, J. W. C. & Francey, R. J. *Science* **269**, 1098–1102 (1995).
- Sarmiento, J. & Bender, M. *Photosynthesis Res.* **39**, 209–234 (1994).
- Bender, M., Battle, M., Sowers, T. & Tans, P. *EOS* **76**, F83 (1995).
- Dixon, R. K. *et al.* *Science* **263**, 185–190 (1994).
- Wofsy, S. C. *et al.* *Science* **260**, 1314–1317 (1993).
- Bazzaz, F. A. *Rev. Ecol. Syst.* **21**, 167–196 (1990).
- Friedlingstein, P. *et al.* *Globi biogeochem. Cycles* **9**, 541–556 (1995).
- Schindler, D. W. & Bayley, S. E. *Globi biogeochem. Cycles* **7**, 717–733 (1993).
- Dai, A. & Fung, I. *Globi biogeochem. Cycles* **7**, 599–609 (1993).
- Houghton, R. A. *Globi biogeochem. Cycles* **7**, 611–617 (1993).

PCR identification of black caviar

SIR — Sturgeons (family Acipenseridae) and paddlefishes (family Polyodontidae), well known as producers of caviar, are threatened by unregulated overfishing, dams eliminating access to spawning grounds, and pollution¹⁻³. Three species inhabiting the Volga River–Caspian Sea basin — the beluga sturgeon (*Huso huso*), Russian sturgeon (*Acipenser gueldenstaedti*) and sevruga (*A. stellatus*) — are particularly vulnerable because of the great demand for their eggs. According to the estimate of the oldest European caviar trading company Dieckmann & Hansen, in 1995 the international market need was 450 tonnes of black caviar, whereas the legal production of caviar in Russia and Iran was 228 tonnes. Even

more telling are figures from the US Commerce Department indicating that caviar imports have increased by 100% since 1991. As things stand, a mere 30 grams of beluga caviar costs \$69.50 at Petrossian in New York City.

As a result, the population sizes of commercial sturgeon species have fallen dramatically during the past few years¹⁻³. Indeed, it has been suggested⁴ that “the sturgeon’s only chance of survival may be in captivity.”

Traditionally, caviar dealers have relied on crude factors such as egg size and the appearance, smell, texture and colour of the roe to identify the species of a particular shipment of caviar. Protein electrophoresis has been used for species diagnosis of caviar, but it requires a large quantity of eggs and is not always accurate⁵⁻⁷. Here we describe the use of the polymerase chain reaction (PCR) for species diagnosis, which allows accurate and relatively inexpensive identification using single eggs.

We examined all extant acipenseriform species for portions of mitochondrial cytochrome *b*, 16S ribosomal DNA and 12S rDNA nucleotide sequences, as well as the variation among individuals from the three commercial caviar species. The data include several unique nucleotide positions that can be used to identify species within this group. We designed several primers specific for diagnostic nucleotide substitutions for each of the three commercial Russian sturgeon species mentioned above. Details of the primer sequences will be pre-

sented elsewhere. We designed the primers by the PCRASS method⁸ so that a high-stringency PCR reaction with primers specific for a given species gives a positive reaction (a band of the correct length on an agarose gel) and nonspecific primers give a negative reaction (see figure).

The table shows the results of our survey of 23 commercially available lots of caviar purchased in reputable Manhattan gourmet food shops and two brought from Russia. We identified five misrepresentations among the New York samples. The labels on the Russian lots did not identify the species (lots 18 and 19 in the table). Three of the misrepresentations are alarming in the light of the fragile status of the substituted fish¹. The appearance of Siberian sturgeon, *A. baerii*, caviar (lot 12) is of concern because poaching in Siberia has increased dramatically. Before 1991, the regulated annual catch of this species was 200–300 tonnes for all Siberian rivers. In 1994 in the Ob River alone, the illegal catch of Siberian sturgeon was about 250–300 tonnes⁹. Our data (lot 4) also show that caviar of the Amur River sturgeon, *A. schrenckii*, which is on the IUCN Red List of threatened animals, and ship sturgeon, *A. nudipectus* (lot 3), are sold in New York. This species is already extinct in the Aral Sea and is rapidly declining in the Caspian Sea¹.

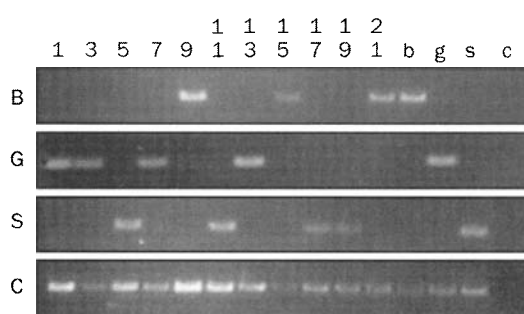
For the members of the order Acipenseriformes, species identification is essential for survival. The use of our method to identify the origin of caviar samples could assist international conservation and legal efforts to save what little is left of the commercial sturgeon populations and help to protect the other species that will

SPECIES DESIGNATIONS FOR 25
COMMERCIALY AVAILABLE LOTS OF CAVIAR

Caviar lot no.	Designation by supplier (trade name)	Diagnosis
1	American sturgeon	Osetra*
2	Caspian beluga	Beluga
3	Caspian osetra	Ship sturgeon*
4	Eastern beluga	Amur River sturgeon*
5	Caspian sevruga	Sevruga
6	Fresh beluga malossol	Sevruga*
7	Fresh osetra malossol	Osetra
8	Fresh sevruga malossol	Sevruga
9	Caviar Russ (beluga)	Beluga
10	Russian caviar (osetra)	Osetra
11	Sevruga malossol	Sevruga
12	Beluga malossol	Siberian sturgeon*
13	Osetra malossol	Osetra
14	Sevruga malossol	Sevruga
15	Beluga malossol	Beluga
16	Osetra malossol	Osetra
17	Sevruga malossol	Sevruga
18	Sturgeon caviar	Osetra
19	Russian caviar	Sevruga
20	Sevruga malossol	Sevruga
21	Beluga prime	Beluga
22	Osetra malossol	Osetra
23	Beluga malossol	Beluga
24	Osetra malossol	Osetra
25	Sevruga malossol	Sevruga

Most lots were identified unambiguously. Lots marked with an asterisk indicate inaccurate labelling by the supplier. Lots 4 and 12 gave negative results in our experiments and were identified by sequencing the positive-control PCR fragment. Lots 1, 9, 13 and 16 were identified both by the PCRASS method and by sequencing the positive-control PCR fragment. Lot 3 gave a positive reaction for one of our *A. gueldenstaedti* primer pairs, but a negative signal for the other and was subsequently diagnosed by sequencing. Ship sturgeon is the common name for *A. nudipectus*, Amur River sturgeon the common name for *A. schrenckii*, and Siberian sturgeon the common name for *A. baerii*. There are three different spellings for osetra on suppliers' labels (Osetra, Osetra and Ossetra). Malossol means 'lightly salted'.

Performance of the PCR assay on DNA isolated from single eggs of commercially available caviar. Initially, DNA from five to ten individual eggs was isolated¹⁰ for each commercial lot. We show only one representative from each odd-numbered lot, as all eggs from a lot gave the same results. The odd numbers (1–21) refer to the commercially available lots to which we gave arbitrary numbers. Results for even-numbered lots are not shown, but are reported in the table. PCR products using beluga- (panel B), sevruga- (S) and osetra- (G) specific primer pairs are shown in this figure. A fourth primer pair was used as a positive control and was designed to amplify all sturgeon samples (panel C). Note that none of the lots gave positive reactions for more than one of the diagnostic primer pairs (B, G or S). These results are verified using other primer pairs and, in some cases, the double-stranded fragment produced by the positive control (panel C) for these four lots was purified and sequenced using an ABI Model 373 automated sequencer. Several positions in this fragment are diagnostic for other species, and these nucleotide positions were used to type these four lots (table). Sample 3, although showing a positive reaction in panel G, did not show a positive reaction for the second *A. gueldenstaedti* primer pair. The control PCR product was therefore sequenced and used to diagnose this lot of caviar. Samples 4 and 12 were the only samples that were negative for all three primer pairs but positive for the control primers. Control PCR of these three lots of caviar as well as lots 1, 9, 13 and 16 were GeneCleaned and sequenced to determine the source species used for making these caviars (see table).



no doubt be more widely substituted for the disappearing commercial caviar species. At present, these commercial species are not included in the appendices of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), the only international agreement on endangered species trade. CITES will consider placing all sturgeon species in the appendices at a meeting (23–27 September 1996) in Pruhonice, Czech Republic. The enforcement of such a listing depends largely on the ability to diagnose these species accurately. Our method will give wholesalers an alternative to the crude identification methods they use now and give consumer groups a weapon to ensure accurate labelling.

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1. Birstein, V. J. *Conserv. Biol.* **7**, 773–787 (1993).
2. Bemis, W. E. & Findley, E. K. *Nature* **370**, 602 (1994).
3. *Sturgeon Q.* **3**(4), 1 (1995).
4. Dumont, H. *Nature* **377**, 673–674 (1995).
5. Rehbein, H. Z. *Lebensmittel Unters. Forsch.* **180**, 457–462 (1985).
6. Keyvanfar, A. et al. *C. r. hebdomadaire. Séances Acad. Sci., Paris* **304**, Ser. III, 191–193 (1987).
7. Keyvanfar, A. *Ann. Inst. océanogr.* **64**, 25–64 (1988).
8. Amato, G. & Gatesy, J. in *Molecular Approaches to Ecology and Evolution* (eds Schierwater, B. et al.) 215–226 (Birkhäuser, Basel, 1993).
9. Ruban, G. *Sturgeon Q.* **4**(1), 8–10 (1996).
10. DeSalle, R., Williams, A. K. & George, M. *Meth. Enzym.* **224**, 176–204 (1993).

Red/blue chaotic power spectra

SIR — Cohen¹ has reported that the time series of chaotic, single-species ecological models have blue power spectra rather than the reddened ones associated with natural populations². In other words, the dynamic behaviour of real populations is apparently dominated by longer-term trends, but population models, of one common type at least, fail to capture this crucial characteristic, being dominated instead by shorter-term responses. This calls into question both the usefulness of the models and the applicability of chaotic dynamics to natural systems. However, equivalent analyses (see Fig. 1) of chaotic metapopulation time series from explicitly spatial models (comprising coupled-map-lattices of $n \times n$ identical patches, describing host–parasitoid³ or host–pathogen⁴ dynamics, with the eight nearest-neighbouring patches linked by dispersal) give rise to distinctly reddened spectra — like the natural population data, but quite unlike the simpler models.

Note that the two-dimensional models within patches are dominated by short-term responses to population density (governed by 'biotic' mechanisms). This tends to undermine any suggestion^{1,2} that the important contrast is necessarily between systems dominated by biotic

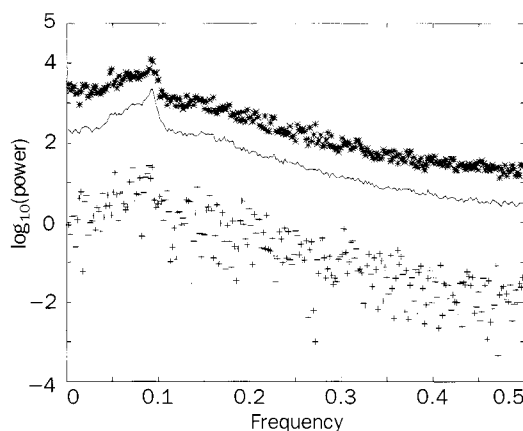


FIG. 1 Power spectral densities of the metapopulation of hosts in a spatially explicit host–parasitoid model³ (similar portraits are observed for the host–pathogen model⁴). Parameter values, taken from ref. 4, are $r = 2$, $\sigma_1 = 0$, $\sigma_2 = 1$, $\Lambda = 1$, $\nu = 1$, $\mu_X = 0.2$, $\mu_W = 0.89$ and $n = 30$; similar results are obtained for larger values of n . In common with natural populations, the spectra are reddened, with greater power at low frequencies. (Scale on axes was chosen to allow comparison with ref. 1.)

factors (blue) and external, 'climatic' factors (red). Moreover, similar spectral analyses of chaotic but non-spatial two-dimensional models^{5,6} still give rise to blue spectra. Thus, we judge that including the spatial dimension is crucial in reddening our power spectra, and that those of natural populations may still be explained (at least partly) by chaotic dynamics.

We can also deduce why, biologically, space can produce reddened spectra. In the spatially explicit lattice, subpopulations respond quickly only to the density in their immediate neighbourhood. Hence, the concerted response of the whole metapopulation to its own total density can occur only on much longer timescales that allow initially local effects to spread and to influence the whole. This echoes, and puts flesh on, the complaints of empiricists that population density may be uninformative because individuals experience only local crowding.

Chaotic population dynamics, when generated by spatially explicit systems, may be important in explaining the reddened spectra seen in natural populations.

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SIR — We wish to add to the debate about colour in the power spectra of the complex dynamics of natural populations^{1,2,7,8}. Power spectra of population trajectories are observed to be white (no dominating frequency) or red (dominance of low-frequency fluctuations^{2,9}). Thus, low frequencies might be expected to dominate in population models producing chaos-like

oscillations. But Cohen showed in various nonlinear models that high frequencies dominate in their chaotic fluctuations¹, making the power spectra blue. Here we report that adding delayed density dependence^{10–12} to these population models reduces the dominance of high-frequency oscillations and either whitens or reddens their power spectra.

In a non-delayed density-dependent system, the population size P_t at time t defines the population size at time $t+1$ with a given growth rate r . We analysed the power spectra of chaotic trajectories of six of the eight models discussed by Cohen¹ by incorporating delayed density dependence into their dynamics. As an example, the Moran–Ricker discrete-time nonlinear dynamics^{13,14}, $P_{t+1} = P_t \exp[r(1 - P_t)]$, modifies into $P_{t+1} = P_t \exp\{r[1 - (P_t + cP_{t-1})]\}$. Of the

models analysed by Cohen, we omitted the Verhulst model, known to produce white noise¹, and the Malthus–Condorcet–Mill model, in which delayed density dependence creates typically isolated spikes in spectra.

It is generally expected that the addition of time delays in population models will lower the stability of the dynamics. However, the consequences of delayed density dependence for the colour of the power spectra of chaotic population dynamics are not known. Adding delayed density dependence in the models can remove the dominance of high-frequency oscillations and can whiten or redden their power spectra (see Fig. 2), which brings them better into line with data from natural populations⁹.

Simple population models have drawn attention to the possibility of complex dynamics in natural populations^{7,8}, but they may be of limited use in explaining the rich phenomena observed in dynamics of natural populations. Chaos in models of natural populations can be obtained in several ways, including periodic doubling (used in simple models¹), quasiperiodicity and intermittency^{15,16}. Depending on the way in which chaos is obtained, there may be fundamental differences in the power spectra of the population trajectories. For example, the quasiperiodic route, which strongly affects the power spectra of the population trajectory, occurs often in the context of species or population interactions, or in age-class interactions in a single population. Delayed density dependence may also produce the quasiperiodic route to chaos. Thus, when adding delayed density dependence to population models, we not only altered the assumptions on the density dependence, but also increased the dimension of the dynamics and moved to

Radiation doses from Ural region

SIR — Electron paramagnetic resonance (EPR) using tooth enamel is a well-known method for measuring external exposure to γ -rays retrospectively. This method is based on determining the concentration of radiation-induced radicals in hydroxyapatite, a mineral component of teeth and bones. It has already been used to deduce the exposure doses of survivors of the atomic bombs of Hiroshima and Nagasaki¹, and of the Chernobyl accident¹, as well as for monitoring doses to nuclear workers in the south Urals². EPR dosimetry has also been proposed for measuring doses of bone-seeking radiopharmaceuticals³. Nevertheless, EPR tooth-enamel dosimetry has not previously been applied to the reconstruction of doses from a combination of internal and external exposure.

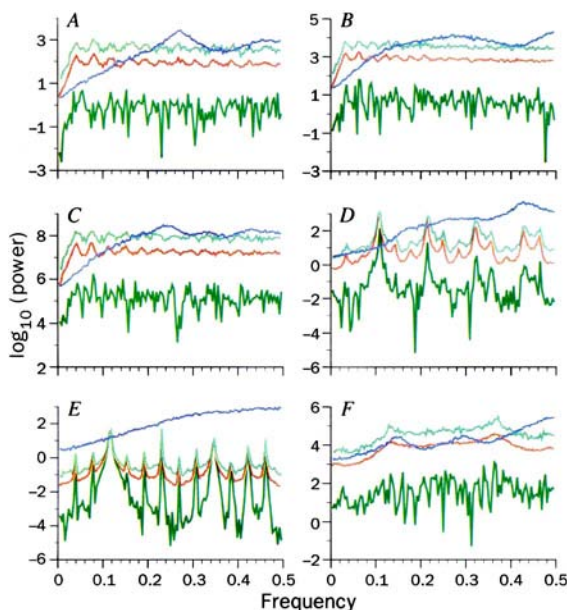
Since 1948, the population of the Techa river valley in the Urals has been exposed to a massive amount of waste from the Mayak nuclear facility near the source of this river. During 1949–51, about 76×10^6 m³ of radioactive waste water containing a total activity of 10^{17} Bq was discharged into the Techa river. Strontium-90 (⁹⁰Sr) and caesium-137 (¹³⁷Cs) respectively contributed 11.6 and 12.2% to the radioactivity. Consumption of the river water caused considerable internal overexposure of the population of the Techa river valley and an excessive incidence of leukaemia⁴.

Dose reconstruction for the Ural region is very important for epidemiological studies. ⁹⁰Sr substitutes for calcium in bone tissues and can accumulate in the body over a long period; its biological half-life is about 20 years. Around 28,000 people lived on the Techa river banks during the period of this massive release. About 4,500 of them were living in the upper Techa riverside, where the level of internal and external exposure was very high. Individual doses due to ⁹⁰Sr can be estimated from measurements of body burdens using a whole-body counter⁵. During the 1970s, individual measurements of ⁹⁰Sr in the skeleton were carried out on 12,248 people (about half the population living in this region during 1949–52). Many people have been examined up to 35 times. The maximum value of the ⁹⁰Sr body burden found for one individual is 0.23 MBq (6,206 nCi). However, there are no reliable data about individual doses resulting from external exposure.

We report here two new approaches to dose reconstruction, which we have applied to survivors of this radioactive exposure in a pilot study.

The first approach is based on the fact that the tooth enamel of an adult human is an unchanging, highly mineralized tissue (97% hydroxyapatite), which does not metabolize ⁹⁰Sr, whereas dentine, which

FIG. 2 Power spectral densities (red line, average of 100 simulations; light and dark green lines, maximum and minimum values observed) of six models with delayed density dependence. Panel A, Moran-Ricker: $P_{t+1} = P_t \exp\{r[1 - (P_t + cP_{t-1})]\}$; $r = 3.7$, $c = 6$. Panel B, Pennycuik: $P_{t+1} = rP_t/[1 + \exp\{-a(1 - (P_t + cP_{t-1})/b)\}]$; $r = 50$, $a = b = 0.1$, $c = 8$. Panel C, Hassell: $P_{t+1} = rP_t/[1 + a(P_t + cP_{t-1})^b]$; $r = 55$, $a = 0.0001$, $b = 100$, $c = 8$. Panel D, Maynard Smith-Slatkin: $P_{t+1} = rP_t/[1 + \{a(P_t + cP_{t-1})\}^b]$; $r = 5$, $a = 0.5$, $b = 4$, $c = 18$. Panel E, Varley: $P_{t+1} = rP_t$ if $(P_t + cP_{t-1}) \leq C$, $P_{t+1} = rP_t(P_t + cP_{t-1})^{-b}$ if $(P_t + cP_{t-1}) > C$; $r = 4$, $b = 3$, $C = 1$, $c = 43$. Panel F, Austin-Brewer: $P_{t+1} = P_t\{1 + r[1 - \exp\{-s(P_t + cP_{t-1})\}]\}K - (P_t + cP_{t-1})\}$; $r = 0.06$, $s = 0.13$, $K = 45$, $c = 0.011$. By contrast with the earlier results³ (blue line), low-frequency oscillations dominate in the models. In each run, the model was simulated for 512 generations to remove the initial transient from the time series. Then the next 512 values were subjected to fast Fourier transform and power-spectrum computations. For ease of comparison, we restricted the parameter values to those used by Cohen¹, adding only the delayed density dependence (the patterns presented in panels D and E can be modified to match those in the other panels more closely by changing parameter values).



a qualitatively different route to chaos. At the same time, we inherited chaotic solutions with different properties and, in particular, different, reddened, power spectra.

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SIR — Both the above suggestions are interesting and plausible extensions of classical models of population change. Both can, in principle, redden the power spectra in a way that matches observations from nature, without invoking external forcing by climate or other factors. Both are important theoretical results. But, in the case of the Chinese locusts^{2,17}, it seems equally likely that Ma's hypothesis involv-

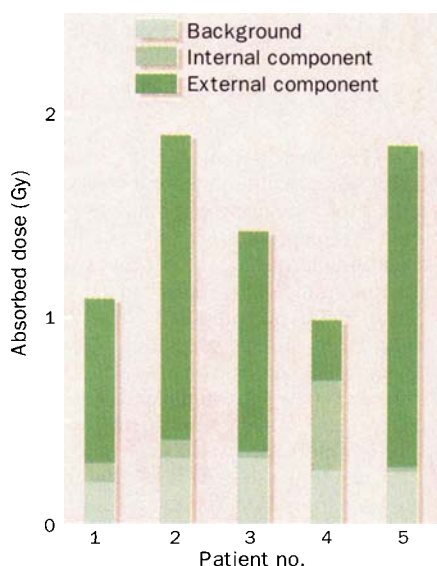
ing climate effects is the main cause of the higher variability seen at the low frequencies there.

All this discussion exemplifies the difficulty of the debate over whether natural population fluctuations represent regulation by internal biological mechanisms or whether they are mostly the result of external environmental forcing^{1,2}. Furthermore, it points to the unlikelihood that this classical dilemma between the climate and biotic schools of population regulation will ever be resolved by any simple (for example, linear) study of the phenomenology of population change. Rather, I believe that this debate points to the need to look deeper: either to more fundamental studies of the basic biology involved, or to sharper quantitative methods that can expose the specific biological mechanisms involved in generating a complex population time series.

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- Cohen, J. E. *Nature* **378**, 610–612 (1995).
- Sugihara, G. *Nature* **378**, 559–560 (1995).
- Comins, H. N., Hassell, M. P. & May, R. M. *J. Anim. Ecol.* **61**, 735–748 (1992).
- White, A., Begon, M. & Bowers, R. G. *Proc. R. Soc. B* **263**, 325–332 (1996).
- Beddington, J. L., Free, C. A. & Lawton, J. H. *Nature* **255**, 58–60 (1975).
- Hénon, M. *Commun. math. Phys.* **50**, 69–77 (1976).
- May, R. M. *Science* **186**, 645–647 (1974).
- May, R. M. *Nature* **261**, 459–467 (1976).
- Halley, J. M. *Trends Ecol. Evol.* **11**, 33–37 (1995).
- Turchin, P. *Nature* **344**, 660–663 (1990).
- Kaitala, V., Ranta, E. & Lindström, J. *J. Anim. Ecol.* **65**, 249–251 (1996).
- Ranta, E., Kaitala, V., Lindström, J. & Lindén, H. *Proc. R. Soc. B* **262**, 113–118 (1995).
- May, R. M. & Oster, G. F. *Am. Nat.* **110**, 573–590 (1976).
- Moran, P. A. P. *Biometrics* **6**, 250–258 (1950).
- Peitgen, H.-O., Jürgens, H. & Saupe, D. *Chaos and Fractals. New Frontiers of Science* (Springer, New York, 1992).
- Rohani, P., Miramotes, O. & Hassell, M. P. *Proc. R. Soc. B* **258**, 17–22 (1994).
- Ma, S. C. *Acta ent. sin.* **8**, 1–40 (1958).



Results of EPR dose reconstructions for five residents of the upper Techa riverside.

has only 70% hydroxyapatite, does metabolize this radionuclide³. Therefore, in the case of ⁹⁰Sr, dentine is the main source of exposure in enamel. Because of the high density of dentine and enamel, the β -radiation of ⁹⁰Sr has a penetration depth of only 0.6–1 mm. This has been confirmed by our experiments with strontium sources and dentine–enamel powder. Taking into account the fact that the estimated penetration depth of the ⁹⁰Sr radiation (β -rays) is low in bone tissue (in contrast to the greater penetration of external γ -rays), one should expect a considerable difference in doses absorbed by enamel and dentine.

EPR dose reconstruction for a 70-year-old resident of the lower Techa riverside who has chronic radiation disease showed 1 Gy for tooth enamel and 5.5 Gy for root dentine. We measured similar ratios for five other inhabitants. Thus, we can use comparative investigation of enamel and root dentine to determine whether the dose obtained was mostly internal or external. Relevant models of the exposure process can be developed to provide quantitative estimates of internal and external components of individual doses.

The second approach consists of a comparative EPR study of three selected groups of the Ural population with different origins of the doses obtained: (1) 86 residents of the Kamensk-Uralskii city 100 km north of the Techa river; (2) 22 residents of the middle and lower Techa riverside; and (3) 5 people who were living in the upper part of the riverside. Dose–age dependence for Kamensk-Uralskii resi-

dents obtained by EPR dose reconstruction with tooth enamel has enabled us to estimate the annual background dose rate for the Ural region at (4.0 ± 1.5) mGy. The last value includes contributions of the natural external background, internal exposure of the human body, radon, medical examinations, solar ultraviolet and the Kyshtym accident.

Further investigations of the origin of the annual dose rate estimated by EPR seem promising. Results from residents of the middle and lower Techa riverside showed a linear correlation between data obtained by whole-body counter and the results of EPR measurements: $D_{EPR} = AR_{Sr} + B$, where $A = 20$ mGy per kBq and $B = 260$ mGy; R_{Sr} is the ⁹⁰Sr individual body burden. With A constant, this formula is valid if the residents of the middle and lower Techa riverside did not receive an external dose from the discharges. This assumption is confirmed by the fact that dose dependence in this group is not affected by distance from the nuclear facility. B designates the average background dose component for adults in the Ural region and correlates well with the value of the annual dose rate of 4 mGy. On the basis of the relationship obtained and the known ⁹⁰Sr body burden, we can evaluate the internal component of enamel dose obtained by the residents of the upper Techa riverside. Subtraction of background and ⁹⁰Sr components from the total EPR doses measured in these

residents allows us to estimate individual external dose. The figure shows the results of the differentiation of the components of doses obtained by these people.

All the results described here are based on doses absorbed by tooth enamel and dentine. It is not clear how we could translate these values into more convenient doses for estimating internal strontium exposure. Fortunately, the dose determined by EPR for root dentine is likely to be close to that absorbed by bone surface. Therefore, the individual dose absorbed by bone surface could be roughly estimated by multiplying the strontium component (AR_{Sr}) by a factor of five (the external and background components would be unchanged). Finally, on the basis of the above speculations, we can obtain the following estimations of doses absorbed by the bone surface of the five residents of the upper Techa riverside shown in the figure: 1.5, 2.3, 1.6, 2.8 and 1.9 Gy.

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Aerial plankton detected by radar

SIR—For many years, meteorologists using sensitive research radars have observed widespread echoes in precipitation-free areas of the diurnal atmosphere. Although the source of these clear-air echoes has been controversial, recent studies have demonstrated that they are attributable to weakly flying insects that drift with the wind^{1–3}. Such insects, whose dispersal in the atmosphere is governed largely by the wind, have been referred to collectively as the “aerial plankton”^{2–5}.

We used powerful C-band Doppler radars⁶ to study relationships between wind patterns and the spatial distribution of aerial plankton during the Convection and Precipitation/Electrification (CaPE) experiment, which was conducted on the east coast of Florida from 8 July to 18 August 1991. On most days during the summer, a mesoscale sea-breeze circulation⁷ develops in this area as a consequence of the different thermal properties of land and water. Low-level winds early in the morning are typically light and from the west. Daytime heating causes warmed air over the land to expand and rise, generating a high-altitude outflow from the land and a compensating low-level inflow of cooler air (the sea breeze) from over the Atlantic Ocean.

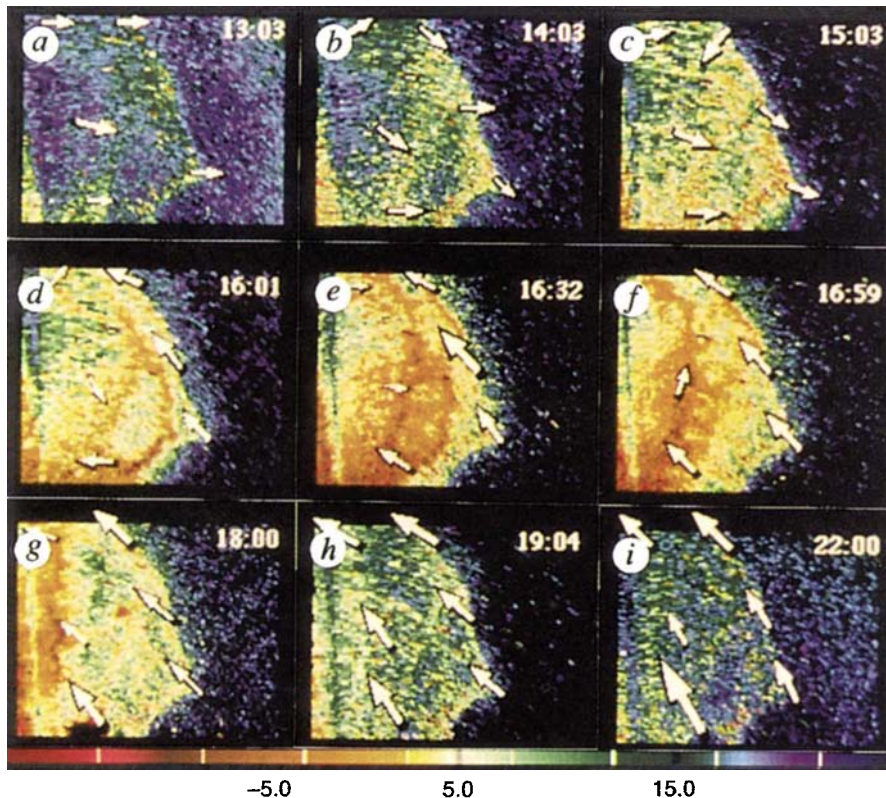
The effects of sea-breeze development on the distribution of aerial plankton were dramatic. The figure shows a time series of radar images for 11 August 1991. In the morning, when the winds blew towards the water, the density of aerial plankton was visibly enhanced along the coastline. Following the onset of the sea breeze, the coastal concentrations were advected westward and dispersed rapidly. Densities of aerial plankton became depressed overall in the afternoon compared with the morning throughout the study area.

The coastal concentrations, together with the absence of overwater echoes

1. Ikeya, M. in *New Application of Electron Spin Resonance — Dating, Dosimetry and Microscopy* 408–417 (World Scientific, Singapore, 1993).
2. Romanyukha, A. A. et al. *Appl. Radiat. Isotopes* **45**, 1195–1199 (1994).
3. Desrosiers, M. F. et al. *Nature* **349**, 287–286 (1991).
4. Kossenko, M. M. et al. *PSR Q*, **2**, 187–197 (1992).
5. Kozheurov, V. P. & Degteva, M. O. *Sci. total Envir.* **142**, 63–72 (1994).

1. Wilson, J. W. et al. *J. Atmos. oceanic Tech.* **11**, 1184–1206 (1994).
2. Russell, R. W. thesis (Univ. California, Irvine, 1994).
3. Russell, R. W. & Wilson, J. W. *Boundary-Layer Met.* (in press).
4. Johnson, C. G. *Migration and Dispersal of Insects by Flight* (Methuen, London, 1969).
5. Drake, V. A. & Farrow, R. A. *Trends Ecol. Evol.* **4**, 381–385 (1989).
6. Keeler, R. J. et al. 25th Int. Conf. Radar Met. 859–862 (Am. Met. Soc., Boston, 1991).
7. Simpson, J. E. *Sea Breeze and Local Winds* (Cambridge Univ. Press, Cambridge, 1994).
8. Heiss, W. H. et al. *Microwave J.* **33**, 79–100 (1990).
9. Crum, T. *Bull. Am. met. Soc.* **76**, 2485–2487 (1995).
10. Brock, F. V. et al. *J. Atmos. oceanic Tech.* **3**, 573–582 (1986).
11. Battan, L. J. *Radar Observation of the Atmosphere* (Univ. Chicago Press, 1973).

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Time series of radar images showing diurnal changes in the low-level (0.3' above horizon) distribution of aerial plankton near Cape Canaveral, Florida, on 11 August 1991. Low-level wind patterns obtained from an automated mesoscale network (mesonet) of surface instrumentation¹⁰ are superimposed as white arrows. The abundance of small insects is proportional to the radar reflectivity factor, which is shown in units of dBZ, and described by the scale at the bottom. Details on radar theory and the technical meaning of the reflectivity factor may be found elsewhere^{1-3,11}. Because of uncertainties about the size distribution of the insects causing radar reflections, the reflectivity measurements shown here cannot be converted directly into density or biomass values, but can nonetheless be interpreted as relative indices of the 'quantity' of aerial plankton in the boundary layer: small quantities are represented by blue shades and large quantities by yellow. (Assuming that the size distribution of aerial plankters was spatially invariant, a difference of 10 dBZ indicates that insect densities differed by about one order of magnitude; a difference of 20 dBZ indicates that densities differed by two orders of magnitude, and so on.) By the start of radar operations in mid-morning (13:03 UTC), the westerly flow had already resulted in some concentration of aerial plankton along the Cape Canaveral coastline (a). The coastal concentration continued to increase until 16:01 UTC, when the sea breeze began to develop (d). After the onset of the sea breeze, the onshore flow advected the aerial plankton westwards. Aerial insect densities were approaching mid-morning levels by 19:04 UTC (h), and by late afternoon (22:00 UTC) most of the radar echoes had been advected westward out of the region. In the last radar image, little contrast is evident between radar reflections over land and those over the adjacent ocean (i).

under all wind conditions, suggest that the radar targets responded behaviourally to landscape features to avoid being drifted out over the ocean. The behavioural mechanisms involved in the avoidance response require further study, but the selective advantage of this response is clear. Terrestrial insects that have drifted to sea probably deplete their fuel stores rapidly and fall to the sea surface. Extensive mortality in these insects should impose strong natural selection for flight behaviours that result in avoidance of overwater drift.

Although largely ignored to date by terrestrial biologists, sensitive Doppler radars have tremendous potential as platforms for conducting basic research on the ecology of aerial plankton. The US National Weather Service is modernizing its radar network, replacing 1950s-vintage

units with new NEXRAD Doppler radars⁸ which have similar capabilities to the radars used in the CaPE experiment. The availability of this technology⁹ should promote interest in the aeolian ecology of aerial plankton, and will eventually allow comparisons of the planktonic faunas of the ocean and the atmosphere.

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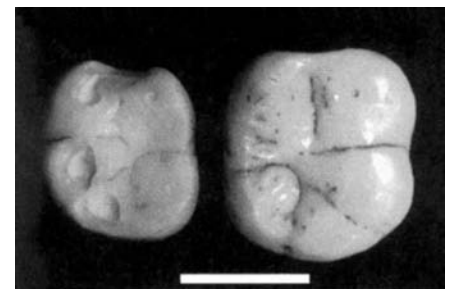
Whose teeth?

SIR — In their interesting and provocative article on the presence of early *Homo* in China, at Longgupo Cave, Huang *et al.*¹ describe the two specimens on which their conclusion is based: a left mandibular fragment with last premolar (P₄) and first permanent molar (M₁), and an isolated upper right permanent second incisor (I₂). Although the stone tools found at the site are certainly evidence of the presence of hominids, we believe that the morphology of the fossils indicates the presence of two hominoids whose specific taxonomic assignments remain unclear.

As the authors state¹, the incisor appears to be hominid in morphology, and in the nature of its deep shovelling and in its crown proportions this tooth may differ somewhat from that of *Homo erectus*. But despite Tobias' view² that "shovelling is a virtually universal feature of all early hominids up to *Homo erectus*", marked shovelling of upper incisors is also characteristic of later species of *Homo* (for example, refs 3–5). Thus, while we agree with Huang *et al.*¹ that the incisor they found is morphologically hominid, the shovelled lingual surface and general crown proportions do not align it with any particular species.

The other teeth found by Huang *et al.* are more problematic. Although the Longgupo molar differs from that of *H. erectus* and resembles *H. habilis* and *H. ergaster* in having five rather than six cusps, this does not signify a special relationship with the latter two species, because the possession of five lower molar cusps is primitive, not only for hominids but for hominoids in general (see ref. 5). Also, while the pattern of wear on the Longgupo M₁ may be reminiscent of that seen in *H. habilis* and *H. ergaster*, as the authors state, the wear pattern and general puffiness and disposition of the cusps of this molar are strikingly similar to those of other hominoids, including an orang-utan-related species from the northern Vietnamese cave site Tham Khuyen⁶ (see figure). Thus, we cannot regard the main features of the Longgupo molar as being hominid apomorphies.

The premolar is also generally hom-



Isolated lower molars (probably first) from Tham Khuyen (TK): left, TK 65/114, left molar, female; right, TK 65/123, right molar, male. Scale bar, 1 cm. (See ref. 6 for further discussion.)

inoid rather than specifically hominid. Thus, although, as the authors state, this specimen may differ from *H. erectus* lower last premolars in being double- rather than single-rooted, double-rootedness is the rule among species of *Homo*, *Paranthropus* and *Australopithecus*, with exceptions restricted to *Ardipithecus* (single-rooted P_4)⁷ (if it is indeed a member of the hominid clade), in the new australopithecine from Chad (three-rooted P_4)⁸, *H. neanderthalensis* (but the roots can be bifid at their tips and bear intraradicular grooves along their broad flanks; see ref. 4) and *H. sapiens* (see ref. 5). Since fossil and extant large-bodied apes also develop double-rooted P_4 s (see ref. 9), the double-rootedness of the Longgupo P_4 could be interpreted as a primitive retention rather than indicative of its phylogenetic relationships or taxonomic identity. Similarly, a relatively large and simple talonid behind the metaconid and protoconid cusps is a feature common to hominoid lower last premolars and is not specific to hominids.

On the basis of the stone tools alone, one can make a case for the presence of hominids in China at whichever date (1 million or 2 million years ago) is eventually confirmed. Morphologically, however, two different hominoids are represented at this site. We eagerly await further evidence that will help resolve their identities.

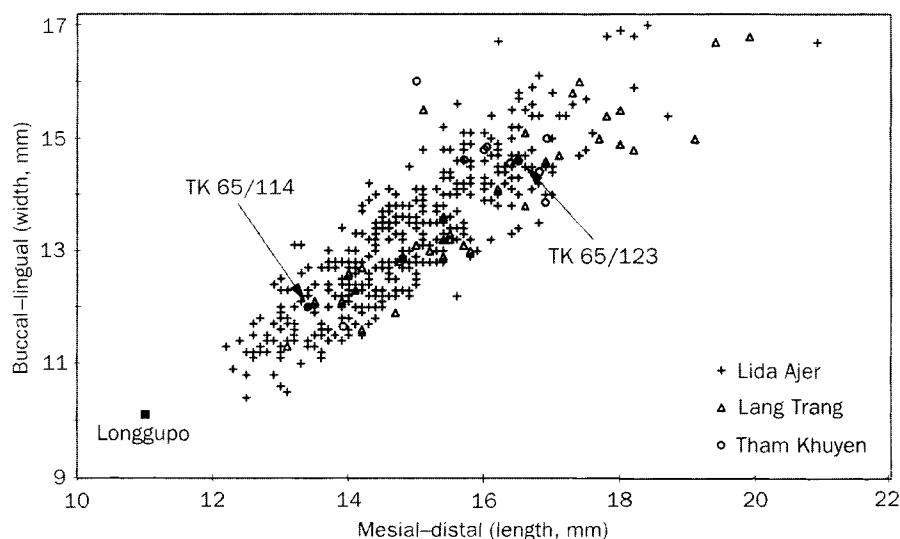
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HUANG ET AL. REPLY — The Longgupo incisor, an unworn permanent tooth from an individual 7 or 8 years of age¹⁰, is remarkable for its shovelling and crown proportions. Hominid incisors can appear shovelled from enlargement of either the marginal ridges or the basal tubercle; mesial-distal curvature; or a combination of these developments¹¹. Therefore, incisor shovelling in extant and extinct hominids is not homologous¹¹. The Longgupo incisor, with heavy marginal ridges, moderate tubercle and light curvature, recalls the condition for Early Pleistocene specimens such as WT 15000 (*Homo ergaster*) and ER 1813 (*H. habilis*). Crown



Lower molar dental metrics for Longgupo¹ and South-East Asian cave sites. The Longgupo M_1 is significantly smaller than the lower molars of *Pongo* from Lida Ajer¹³, Lang Trang¹⁵ and Tham Khuyen⁶ caves, which represent nearly all such teeth from South-East Asia. Data for Lang Trang and Tham Khuyen samples and for TK 65/114 and TK 65/123 were provided by V. T. Long, Institute of Archaeology, Hanoi; data for Lida Ajer and other Sumatran caves were from D. A. Hooijer¹³ and from J. de V.

proportions for the Longgupo incisor fall within the range for OH 6, OH 16, OH 39 and ER 1813 (*H. habilis*)², and just below the two specimens known from Zhoukoudian (*H. erectus*)¹². Finally, the crown axis parallels the root, a pattern observed in *H. erectus*^{12,13}.

For the Longgupo mandible, our discussion of dental apomorphies follows Schwartz and Tattersall's lead in relating the Longgupo molar to their "orang-utan-related species," which they define on the basis of two isolated molars but do not name. We have recently shown that the Tham Khuyen pongid dental assemblages compare morphologically and chronologically to those of Lang Trang, Vietnam, and Lida Ajer, Indonesia^{14,15}. The Longgupo molar is, nevertheless, significantly smaller than any other within this undifferentiated Middle Pleistocene population (see figure). As a consequence, although a five-cusped lower molar and double-rooted last premolar may be hominoid primitive retentions, these features do not align Longgupo with a pongid alternative.

Moreover, the Longgupo cheek teeth show two levels of hominid apomorphy. First, they have thick enamel and vertical buccal surfaces, both undeniably derived hominid features of primary order. Second, the Longgupo molar cusps are

placed peripherally and the fifth cusp is inclined buccally. As the double-rooted premolar is also considerably smaller than similar teeth from all Asian "large-bodied apes," the comparison is again spurious.

Finally, while the premolar's expanded talonid basin may be another hominoid primitive retention, the relationship between the basin, the cusps and the general plan of the premolar is distinctly hominid and compares directly to OH 13 and ER 992. In particular, the two principal cusps are disposed mesially, and the talonid itself has a deep fovea.

Given the fragmentary state and the limited value of an isolated incisor, the full taxonomic status of the Longgupo dental fossils awaits corroborative evidence. However, Longgupo's mandibular features do not recall Middle Pleistocene pongids, and the shovelling of its incisor does recall other Plio-Pleistocene hominids, not *H. sapiens*. With their combination of primitive and derived hominid features and the Plio-Pleistocene age of their cave context, the Longgupo specimens continue to suggest the earliest members of the human clade outside Africa.

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- Huang, W. *Nature* **378**, 275–278 (1995).
- Tobias, P. V. *Olduvai Gorge IV: The Skulls, Endocasts and Teeth of Homo habilis* (Cambridge Univ. Press, 1991).
- de Castro, J. M.-B. *J. hum. Evol.* **24**, 339–371 (1993).
- Wolpoff, W. *Am. J. phys. Anthropol.* **50**, 67–114 (1979).
- Schwartz, J. H. *Skeletal Keys: An Introduction to Human Skeletal Morphology, Development, and Analysis* (Oxford Univ. Press, New York, 1995).
- Schwartz, J. H. et al. *Anthrop. Pap. Am. Mus. nat. Hist.* no. 76, 1–23 (1995).
- White, T. D., Suwa, G. & Asfaw, B. *Nature* **371**, 306–312 (1994).
- Brunet, M. et al. *Nature* **378**, 273–275 (1995).
- Aiello, L. & Dean, C. *An Introduction to Human Evolutionary Anatomy* (Academic, New York, 1990).
- Smith, B. H. in *Advances in Dental Anthropology* (eds Kelley, M. A. & Larsen, C. S.) 143–168 (Wiley-Liss, New York, 1991).
- Crummett, T. L. *The Evolution of Shovel Shaping: Regional and Temporal variation in Human Incisor Morphology*. (Univ. Microfilms, Ann Arbor, 1994).
- Weidenreich, F. *Paleont. sin. n.s.* **D1**, 1–180 (text) & 1–121 (atlas) (1937).
- Hooijer, D. A. *Zool. Med. Mus. Leiden* **29**, 175–301 (1948).
- Ciochon, R. et al. *Proc. natn. Acad. Sci. U.S.A.* **93**, 3016–3020 (1996).
- Long, V. T., de Vos, J. & Ciochon, R. *Indo-Pacif. Prehist. Ass. Bull.* **14**, 119–128 (1995).

No place to hide

John Massey Stewart

Environmental and Health Atlas of Russia. Editor-in-chief Murray Feshbach. *PAIMS: 1995. Distributed by the Center for Post-Soviet Studies, 2 Wisconsin Circle, Suite 410, Chevy Chase, Maryland 20815, USA. Pp. 448. \$95 (pbk).*

Ecological Disaster: Cleaning up the Hidden Legacy of the Soviet Regime. By Murray Feshbach. *Twentieth Century Fund: 1995. Pp. 157. \$9.95 (pbk). Distributed by the Brookings Institution.*

'ECOCIDE' is the word coined by Murray Feshbach, ex-Sovietologist and research professor of demography at Georgetown University, Washington DC, in the seminal book he wrote with Alfred Friendly, *Ecocide in the USSR* (BasicBooks/Aurum, 1992). It refers to the self-inflicted twin disasters of environment and health facing the Soviet Union at the end of the Communist era. Feshbach has now produced a detailed atlas of post-Soviet Russia's environment and health, and an updated survey of Russia's environmental problems and threats, with concrete suggestions for Western collaboration.

The atlas has been compiled by a large team of Russian experts with US support. In his foreword Aleksei Yablokov, chairman of the Russian Security Council's Interdepartmental Commission on Environmental Security, suggests, surely correctly, that the atlas is unique in being the most advanced attempt to compare data on health and environment in such an enormous territory. He points out that whereas ecological information was confidential before *perestroika*, a 1993 law on state secrets now calls for full publication of all environmental and health data.

The 304 maps, coloured by province and supported by the text, portray demographic changes from 1989 to 1992, environment-cum-geography and finally public health. They show regional comparisons between all 76 provinces (including constituent republics) and cover a huge range of subjects, from atmospheric pollution emissions and surface-water quality to the incidence of rare plants and forest fires, pesticide storage to be destroyed or buried — a new and urgent problem — and on to radioactive pollution sites in the Moscow *oblast* (administrative region) and radioactive accidents and incidents in 1993. In that year, 200 operational violations were recorded at Russia's nuclear power stations.

The hundred or so health maps, far more abundant than their environmental counterparts, cover infant mortality and respiratory illness, the incidence of many individual diseases, life expectancy and

potential years of working activity lost because of premature mortality from illness and disease — although regrettably there is nothing on congenital abnormalities which could show priority areas for environmental improvement. According to the text, life expectancy in Russia has dropped steadily to 71.9 years for women and to 58.5 for men, two out of three fetuses are artificially aborted and abor-

Losing ground: storage point for contaminated trucks in the Chernobyl exclusion zone.

tion causes up to 25 per cent of all maternal deaths. Only 40 per cent of children are born healthy, and infectious diseases have increased alarmingly: between 1992 and 1993, the incidence of child diphtheria and measles increased by around 300 per cent.

The maps give a remarkably instant picture of comparisons across Russia, but such huge provinces as Krasnoyarsk with their marked north-south differences may give only a misleading average. Perhaps a new edition — and in a less cumbersome shape — could add some examples at the lower regional (*rayon*) level, synchronize the text and maps better and provide more interpretation of the maps. One hates to cavil in view of the effort and undoubtedly major achievement — the work will be of great use to many planners and scholars — but analysis, particularly of the correlation between health and the environment, is disappointingly absent. Surprisingly, very few regional patterns stand out from the maps (or text) except the effects of the lack of infrastructure, mostly, it seems, in remote indigenous

republics such as Yakutia and Tuva. And oddly, Moscow and St Petersburg often come out badly; for instance, in those cities, and in five other regions, at least 41 per cent of children were not vaccinated against diphtheria in 1993.

The atlas gives no cause for complacency. The total of all radioactive waste products, for example, is estimated at about 2.8 billion curies and 610 million cubic metres. According to both Russian and World Health Organization standards, the maximum allowable concentration of many substances is exceeded fivefold in more than 150 cities and tenfold in 86 cities, thus affecting 95 million people. Almost all reservoirs close to cities are contaminated and 75 per cent of all surface water is polluted, with only half of all water safe for drinking even after treatment. Interestingly, Chechnya has the lowest number per head of population of doctors, paediatricians, X-ray examinations and hospital beds.

Feshbach's purpose in writing *Ecological Disaster* is to go beyond description and analysis of the environmental and health crisis in the former Soviet Union and to highlight the principal issues that must be addressed and confronted by Russia and the West in consort. He draws valuable attention to the global environmental threats — the Soviet regime's "hidden legacy" — generally unappreciated in the West except for the question of the safety of nuclear power stations: nuclear dumping and decommissioning; radioactivity; the massive chemical and biological warfare facilities built up during the Cold War; the potential 'poisoning' of seas by radioactive and chemical dumping; and the accelerated and unsustainable felling of the great Russian forest with its potentially dangerous contribution to global warming. Ominously, Feshbach points out that the former Soviet Union's nuclear, chemical and biological facilities are not only threatening to the world but are also themselves under threat as potential targets for mafia or terrorist activity.

After an unnerving summary of the major problems, Feshbach focuses on how

Oliver Shirley/EPL

Science Book Prizes

This year's Rhône-Poulenc Prizes for Science Books were announced yesterday at the Science Museum in London. The winner of the £10,000 general prize is Arno Karlen for *Plague's Progress: A Social History of Man and Disease* (Gollancz/Putnam). The junior prize goes to Chris Maynard's *The World of Weather* (Kingfisher; published in the United States by Scholastic as *Wind and Weather*).

the West can help and what policies and priorities it should adopt. I agree that the West has been painfully slow in delivering the goods, has raised expectations ill-advisedly and is seen not only as talking much and doing little but also as feathering its own nest in its aid programmes — all fuelling anti-Western feeling and instability. A huge political prize has been lost.

Feshbach pleads for an international mechanism to reach common decisions and to coordinate efforts for a comprehensive attack on the problems. "To date our efforts to aid Russia and the other states have been remarkably uncertain and uncoordinated", he laments. The problem, he rightly sees, is not simply the level of total Western assistance pledged but the slowness of implementing the aid, the duplication of effort and the absence of any overall strategy or set of priorities.

He urges an annual review of project performance for setting future priorities and pledging the next cycle of financial assistance, and advocates as a model the aid mechanism created for Indonesia in the late 1960s. He makes specific recommendations, albeit directed at the United States only, such as increased remote sensing and the environmental use of Russia's armed forces. And he rightly stresses how important it is that the former Soviet governments should feel they are playing an integral part in the planning process. The lesson is that health and environment problems are not peripheral but a serious national and international security issue. □

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Spectacular explosions

A. G. W. Cameron

Supernovae and Nucleosynthesis: An Investigation of the History of Matter, from the Big Bang to the Present. By David Arnett. *Princeton University Press*: 1996. Pp. 598. \$85, £66.50 (hbk); \$39.50, £27.50 (pbk).

THERE is a parallel between the design of thermonuclear weapons and the attempt to understand supernova explosions. The first challenges the theoretical physicist to make precise calculations that bring together the treatments of extreme conditions in plasma physics, thermodynamics, statistical mechanics and nuclear physics, all within the context of an elaborate hydrodynamic simulation of a device to be built in the workshop. The supernova explosion presents a similar challenge to the theoretical astrophysicist, except that the device is designed and built by nature, and the astrophysicist must deduce its construction as part of an attempt to understand its destruction. David Arnett gives a brief overview of the nuclear physics needed to understand some aspects of nucleosynthesis in the supernova explosion, but he does not attempt to summarize the rest of the relevant physics, and prospective readers should ideally be familiar with all of these topics. By way of compensation, he always emphasizes the physical understanding of the phenomena he discusses, and with appropriate preparation the

book is delightful reading.

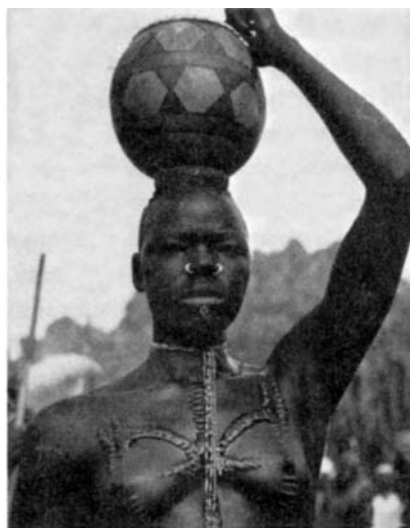
A more conventional treatment of supernova explosions would take a star of given mass and follow it through the various stages of its evolution and explosion, and then do the same for a star of different mass. Arnett prefers to consider different aspects of evolution separately, taking together all the different masses. This may bother some readers who would like to have all aspects of a given type of supernova dealt with in one place, rather than scattered among different topics, although there is also logic in Arnett's approach. Perhaps an electronic edition of this book could be set up with hypertext links, so everyone could be satisfied.

In his preface Arnett writes: "This book is not intended to be a scientific history, a textbook, or a review, although it has some of these elements and could serve these purposes. It is intended to be what was well described by Prof. S. Chandrasekhar in reference to his own goals for scientific books: '...a certain viewpoint of the field, written by one who has been an active participant in its development...'. Arnett has long been one of the leaders in the field of supernova explosions, starting with his PhD thesis a little over 30 years ago; the field has since developed into a small industry. His book emphasizes the areas in which he has worked, in accordance with his overall objective. But some important topics in nucleosynthesis are missing, in particular the formation of the very heavy elements by neutron capture on slow and rapid timescales (the s- and r-processes). Nor is the evolution of stars of medium mass (which do not produce supernovae) discussed in any detail, and it is in the asymptotic giant branch phase of such stars where the s-process takes place. Arnett's final chapter on Galactic evolution can therefore present only part of the general picture of the history of nucleosynthesis.

These omissions are not important in themselves, because Arnett's approach is intentionally selective. But to use the book as a textbook supplement for a course on stellar evolution, a teacher must be prepared to treat the missing topics by referring to other sources (and also to review some aspects of the more advanced physics). That said, the book is the best available source of material on supernova physics for the graduate student. □

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■ The third edition of *A Dictionary of Physics* edited by Alan Isaacs had just been published in paperback by Oxford University Press at £6.99.



Richard Lewontin's *Human Diversity* is now out in paperback in the Scientific American Library series. The author discusses the new techniques of DNA analysis and the results that have appeared since the book was first published in 1982. But his overall conclusion remains the same: that the very aspects of human diversity that fascinate us the most remain outside the domain of genetics. W. H. Freeman, \$19.95, £15.95.

Islands of peril and pleasure

Lawrence B. Slobodkin

Krakatau: The Destruction and Reassembly of an Island Ecosystem. By Ian Thornton. *Harvard University Press*: 1996. Pp. 346. \$39.95, £25.50.

The Song of the Dodo: Island Biogeography in an Age of Extinctions. By David Quammen. *Scribner*: 1996. Pp. 704. \$30.

A CONTINENTAL island results when a water passage cuts off a piece of a mainland, an oceanic island when new land emerges from the sea. Newborn continental islands carry a sample of mainland species and are a natural experiment on the effect of cutting off part of an existing ecosystem. Oceanic islands, lifeless until propagules come across the sea, are an experiment in the invasion and establishment of ecosystems. Species on islands may be conspicuously absent (such as Irish snakes) or peculiar (such as the giant tortoises of Aldabra). Species on islands are singularly prone to extinction. High mountain tops, lakes, tufts of residual forest and oases can be almost as strange, fragile and isolated as islands.

Both Ian Thornton and David Quammen present exciting, information-rich, complementary accounts of island biology. Thornton focuses on the most famous of island volcanic eruptions. Quammen gives his personal view of island biogeography, its facts, theories and people.

On 27 August 1883, Krakatau, between Java and Sumatra, exploded. The sound was heard in Sri Lanka. There were tsunamis higher than a seven-storey building. Earth's atmosphere was loaded with cubic kilometres of dust, temperature was lowered globally and sunsets were reddened for four years. Where there had been green islands lay a mound of ash and lava. Three months later a lone spider was seen building its web, and over the next century the reinvasion of life continued. Krakatau is today again a lush set of tropical islands.

Krakatau's regeneration, species by species, is understandable in terms of basic ecological principles and each species' natural history. Floaters and windborne autotrophs came first, then swimmers and those riders on flotsam that are not hurt by salt water. Food plants preceded herbivores, and carnivores were accepted only later. Extinction or increase was generally independent of population size unless populations were extremely small. New invaders echoed through the ecosystem, opening or closing windows for others. As forests developed, open-land

species vanished while tree lovers such as orchids and snakes established themselves. Thornton, an accomplished biologist, presents a detailed history replete with fascinating details about both the organisms and their investigators.

Quammen is a nonscientist, proudly but unfortunately lacking in mathematical literacy. He summarizes in exciting earthy prose what he learned about island biol-



Krakatau: an experiment in the invasion and establishment of life.

ogy and species extinction during eight years of adventurous travel, reading and conversation. Descriptions of places alternate with chapters on the history of ideas and accounts of conversations with biologists. It was after reading about Alfred Russel Wallace and his relationship with Charles Darwin in J. L. Brooks's excellent and sadly little-read book *Before the Origin* that Quammen went to the islands Wallace visited, which are still well off all beaten tracks. Quammen also visited Madagascar, Amazonia, the island of the Komodo dragons and elsewhere. His vivid and appealing descriptions will surely hasten the day these fabulous locations become the sites of ecotourism hotels.

Quammen's adventure began when he was swept up in the charm of *The Theory of Island Biogeography* by the well-known specialist E. O. Wilson and the late Robert MacArthur (who Quammen describes as "the James Dean" of theoret-

ical ecology). Quammen's lack of theoretical sophistication permitted him to accept the book as a foundation stone of ecological theory rather than as a not entirely fortunate phase of the history of ecology.

The Theory of Island Biogeography is based on several reasonable assumptions (for example, larger land masses are likely to contain more species than smaller ones, immigration is more frequent from nearer sources and the number of successful invasions is proportional to the number of migrants). It also contains several incorrect assertions (such as that all species are interchangeable and extinction probability is always inversely proportional to abundance).

Wilson has reported several times that he, MacArthur and I began collaborating on formal ecology in the late 1950s but that I developed a "hatred for MacArthur" and dropped out, a story Quammen reiterates. It is not true. Privately, MacArthur and I remained friends. (My garden still contains descendants of tulip bulbs he gave me.) But I was, and am, unhappy with the kind of theory that was being produced and felt it intellectually necessary to dissociate myself from it. The experience ultimately forced me to concentrate on the meanings and uses of simplification in science and particularly the distinction between the simplified and the simplistic, an approach I outlined in my book *Simplicity and Complexity in Games of the Intellect* (Harvard University Press, 1992). I abandoned ship when biology lost its place in the theory, and the history of biogeography and conservation biology has, I believe, borne me out.

The gross simplifications in *The Theory of Island Biogeography* allowed simple-looking graphs and elaborate-looking mathematics. The idea was an exciting one; and it took 15 years and a great deal of work, much of it documented by Quammen, to demonstrate that the theory was useless for explaining or predicting actual cases. Quammen's account of the verbal combat and brilliant if colourful combatants involved in reaching this consensus makes great reading. Thornton states that Krakatau did not follow the graphs of *The Theory of Island Biogeography*. Surprisingly, the text of that book itself indicates that Krakatau did not conform to the theory. Also, Quammen's examples of saving threatened species from extinction all hinge on intimate knowledge of natural history rather than

simplistic mathematical generalizations.

Both books provide hours of truly pleasurable reading and also clear and deep insights into the fascinating biology of islands, the struggles to construct comprehensible theories for difficult processes and the techniques for preserving species diversity. They also both introduce a fascinating cast of investigators and entrepreneurs in these fields and islands. Quammen's work, however, must be read with a grain of salt because of his failure to understand the theory and his uncomfortable hagiography of some of the scientists he interviewed. □

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Balancing act

Miles Padgett

Lasers and Electro-Optics: Fundamentals and Engineering. By Christopher C. Davis. Cambridge University Press: 1996. Pp. 720. £80, \$125 (hbk); £29.95, \$44.95 (pbk).

HARDLY a day passes without yet another book appearing on lasers and optoelectronics. Budding authors must strike a balance between heavyweight optical physics and modern device technology. Too much technology and the book is out of date before it reaches the library shelves; too little and it ceases to be relevant to this scientifically fertile and commercially expanding field.

Pitched at specialist undergraduate or graduate students, this book can be warmly recommended. It covers most aspects of laser physics, from basic rate equations to resonator design, is liberally supported by examples of the latest technology and contains a healthy selection of references. There are strong sections on optical systems, including ray propagation, Gaussian beams, fibres and detectors. Towards the end, especially for readers who have found the earlier parts all too easy, there is an excellent chapter on coherence theory.

In a general text such as this, much must be left out. That explains why there is no mention of SEED devices in the parts on technology, or of Stokes parameters in the section on the optical systems, or of optical data storage in the coverage of applications. But these omissions do not detract from the achievement and value of the volume as a whole. □

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Easy learning

Patrick Naylor

Pattern Recognition and Neural Networks. By B. D. Ripley. MIT Press: 1996. Pp. 403. £29.95, \$49.95.

KNOWN for his hype-free approach to neural networks, Brian Ripley here provides an excellent text on the statistics of pattern classifiers and the application of neural network techniques. With an emphasis on the statistical approach, it would have been all too easy for a book of this type to become hard work to read. Ripley has managed, however, to produce an altogether accessible text, aided by examples using both synthetic and real-world data sets. These include case studies on, believe it or not, *Leptograpsus* crabs and diabetes in Pima Indians.

The scope of the book is wide, and incorporates neural networks initially as methods for learning the parameters of

multi-dimensional input-output relationships; only later on does Ripley make passing reference to any architectural equivalence to the brain. The opening chapter on basic decision theory and Bayes rule is substantial and leads to a development of parametric models. Other chapters cover a range of topics such as linear discriminant analysis, multilayer perceptrons and radial basis function networks, as well as unsupervised methods.

The inevitable comparison between this book and C. M. Bishop's *Neural Networks for Pattern Recognition* (Oxford University Press, 1995) suggests that while Bishop's book is earning a reputation for depth and rigour, Ripley's text will be rightly popular with newcomers to the area for its ability to present the mathematics of statistical pattern recognition and neural networks in an accessible format and engaging style. □

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IRIDESCENCE, a widespread but rare optical effect that usually occurs in winter over mountains, is caused by diffraction of light from the Sun or Moon by a thin layer of middle-level cloud of uniform droplet size. The picture is taken from *Weather* edited by Richard Whitaker, which claims to be "a comprehensive and accessible introduction to meteorology, its past, present and future". Particularly useful is the well-illustrated hundred-page field guide to common weather and atmospheric phenomena. Collins/Time-Life Books & The Nature Company, £14.99, \$29.95.

Cosmological baryon density derived from the deuterium abundance at redshift $z = 3.57$

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THE primordial ratio of deuterium to hydrogen nuclei (D/H), created as a result of the Big Bang, provides the most sensitive measure of the cosmological density of baryons^{1–5}. Measurements of the D/H ratio in the interstellar medium of our Galaxy place a strict lower limit on the primordial ratio⁶, because processing of gas by stars reduces the abundance of deuterium relative to hydrogen. Absorption of radiation from distant quasars by intervening clouds of gas offers a means of probing D/H ratios at large redshifts, where the effects of stellar processing should be negligible. Measurements^{7,8} on one absorption system have indicated an extremely high primordial abundance ratio of 24×10^{-5} . Here we report a measurement of the D/H ratio in another high-redshift absorption system, and obtain a value that is an order of magnitude lower than that reported previously^{7,8}. The measured ratio of 2.3×10^{-5} is consistent with that in the interstellar medium (after allowing for Galactic chemical evolution^{9,10}), and indicates that the absorption spectra on which the earlier estimates are based may have been subject to strong contamination. We calculate a baryon density that is 5% of the critical density required to close the Universe.

Deuterium is rarely seen in quasar absorption systems because the associated hydrogen is usually distributed over a wide range of velocities and its absorption spectrum obscures that of deuterium. We selected quasar 1937–1009 because we found that the absorption system at $z = 3.572$ showed unusually weak metal lines in low-resolution spectra, which we took to indicate that the gas was distributed in one or two narrow velocity components¹¹. We obtained optical spectra of this quasar ($z = 3.78$, $V \approx 17.5$ mag) with the HIRES echelle spectrograph¹² on the W. M. Keck 10-m telescope. The high-quality spectra make this amongst the best characterized quasar absorption systems, and show strong absorption at the expected position of the D Ly α line and weak but highly significant absorption at D Ly β .

The weak metal lines in the system, C II, C IV, Si II and Si IV, appear to have the same profiles, which are adequately described by two components separated by 15 km s^{-1} . The velocity positions determined from the metal lines (Fig. 1) lie in the cores of the higher-order Lyman lines (Fig. 2), and at the expected positions of the deuterium absorption features. The absorption features are modelled assuming that the velocities of these two components, labelled blue (zero velocity) and red, are common to every ion. The parameters (and their statistical errors) which produce the smooth curves in Figs 1 and 2 are shown in Table 1.

The Lyman lines show that $> 90\%$ of the H I must be in, near or between the two components, but not elsewhere because there are no other lines in the spectrum which can explain the Lyman limit. A simultaneous fit to the two components gives a total column density $\log N(\text{H I}) = 17.94 \pm 0.05$. D/H will change if the total H changes, but not if the H is redistributed in the velocity range where the D is measured, because D is unsaturated. There could be additional H at velocities between, or near these components, which would need $b < 20$ ($b \equiv \sqrt{2}\sigma$ is a measure of the intrinsic, not instrumental, line width): But this can not significantly increase the total $N(\text{H I})$, because the wings of Ly α require $\log N(\text{H I}) < 18.04$. We can not decrease the total $N(\text{H I})$ in our model because the cores of the high-order Lyman lines, and the

Lyman continuum, must be completely absorbed. An additional component at $\sim +49 \text{ km s}^{-1}$ produces Si IV, C IV and H I absorption, but it is absent from the high-order Lyman lines, it has a low column density of $\log N(\text{H I}) = 14.94 \pm 0.14$, and it cannot change D/H.

The red D component is blended with both the blue D and H components, so its parameters are poorly constrained by the data. We fit both components simultaneously and require that $(\text{D}/\text{H})_{\text{red}} = (\text{D}/\text{H})_{\text{blue}} = (\text{D}/\text{H})_{\text{total}}$. We also restrict its b value to the range allowed by $b(\text{H})$. The total D in both components is $\log N(\text{D I}) = 13.30 \pm 0.04$, which is fairly insensitive to $b(\text{D})$ and the precise velocity of the D because the D lines are unsaturated.

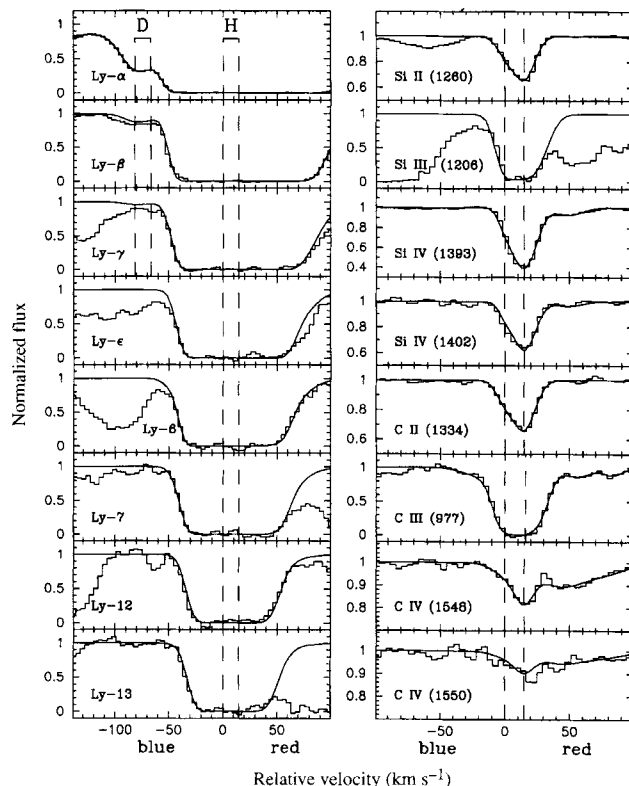


FIG. 1 Velocity plots of Lyman-series lines (left), and all the metal lines (right) in the absorption system towards quasar 1937–1009. Zero velocity, indicated by a dashed line in all plots, corresponds to the redshift $z = 3.572201$ of the blue component. The red component at $z = 3.572428$ is indicated by a second dashed line at $+15 \text{ km s}^{-1}$. The deuterium lines lie 82 km s^{-1} to the blue of their respective hydrogen lines. The histogram represents the observed counts of the combined Keck spectra in each pixel, normalized to the quasar continuum. The smooth curve shows the Voigt profiles convolved with the instrumental resolution which produces the best simultaneous fit to all the lines. The values and 1σ errors which characterize the smooth curve are summarized in Table 1. Levshakov and others^{33,34} have developed the mesoturbulent theory which gives absorption line profiles that differ from normal Voigt profiles when $b_{\text{tur}} \geq b_{\text{therm}}$ and the turbulent velocity fields are correlated on distances less the cloud size. In the limit when thermal velocities (which are necessarily uncorrelated) dominate, mesoturbulence predicts the Voigt profiles that we have used. In all hydrogen and deuterium lines of our system $b_{\text{tur}} < 0.4b_{\text{therm}}$, so we conclude that Voigt profiles are adequate. The Lyman series lines, C III (977 Å), Si II (1,260 Å) and Si III (1,206 Å) are all in the Lyman- α forest region where there is abundant absorption, usually from cosmologically distant and unrelated H I. We do not fit this additional absorption, which is seen in Si III (1,206 Å) and D Ly β , and we omit the most blended Lyman lines. The signal-to-noise per pixel ($\sim 4 \text{ km s}^{-1}$) for the Lyman series from top to bottom is 75, 31, 22, 19, 17, 15, 15 and 13. For the metal lines, from top to bottom, it is 100, 20, 71, 40, 56, 23, 50 and 50.

We then have $\log D/H = -4.64 \pm 0.06$, where the error includes random photon noise and fitting errors, but not systematic errors.

Statistically we expect some H in the region of the D lines, because weak H I absorption is extremely common. This will systematically increase $N(D)$. We generated noise-free model spectra for various $N(D)$, using the redshifts, b -values and the $N(H I)$ determined above. To each spectrum we then added Ly α forest lines with random N , b and z values drawn from known distributions (ref. 13, and D. Kirkman and D.T., manuscript in preparation). For a given D/H, we made 500,000 random spectra, and we calculated the likelihood that the data came from the realizations of that model. The maximum likelihood occurred for the measured D/H with no significant Ly α contamination. The likelihood function has a long tail to lower D/H with an expected (mean) value of $D/H = (2.27^{+0.09}_{-0.14}) \times 10^{-5}$, (1σ errors from H blended with $N(D)$, and photon errors at D), only slightly lower than the D/H of maximum likelihood. We conclude that in this quasar absorption system, random Ly α lines are unlikely to give profiles that look like the observed D, and that the absorption we measure is entirely deuterium (to within the random errors).

We estimated additional systematic errors from the uncertainty in the continuum level by moving the continuum $\pm 2\%$ at the Ly α feature, and $\pm 5\%$ at the Lyman limit. These percentage adjustments correspond to the 1σ error per pixel in each region of the spectrum. The random error in the continuum level should be much less than this, because the continuum is constant over many pixels. The same fitting procedure was run with each new continuum level. When the continuum was raised in both regions we found $\Delta \log N(H I) = +0.04$ and $\Delta \log N(D I) = -0.02$, and when it was lowered, $\Delta \log N(H I) = -0.03$ and $\Delta \log N(D I) = +0.02$. We conclude that $\log(D/H) = -4.64 \pm 0.06^{+0.05}_{-0.06}$, or $D/H = (2.3 \pm 0.3 \pm 0.3) \times 10^{-5}$, where the log form is more accurate and the errors represent the 1σ statistical uncertainty, followed by the systematic uncertainty.

As a consistency check, we used the measured b values of the C II, Si II and hydrogen lines to find the temperature and turbulent velocity of each component. We assume that all lines of these ions have the same turbulent widths, b_{turb} , and temperature T . The intrinsic line widths are $b_{\text{total}} = (b_{\text{therm}}^2 + b_{\text{turb}}^2)^{0.5}$, where the thermal widths depend on the mass of each ion: $b_{\text{therm}} = 0.128(Tm_p/m_{\text{ion}})^{0.5} \text{ km s}^{-1}$, where m_p is the proton mass, and values are given in Table 1. We then predict that the blue component of D has $b = 12.5 \pm 2.1 \text{ km s}^{-1}$, which is consistent with the measured value of $b(D I) = 14.0 \pm 1.0 \text{ km s}^{-1}$.

We use the column densities of the metal ions to deduce the metallicity and neutral fraction in the absorbing gas. Following Donahue and Shull¹⁴, and assuming a typical quasar photoionizing spectrum given by Mathews and Ferland¹⁵, we estimate the ionization parameter (the ratio of the number of photons with energies above one Rydberg to the number of atoms: n_γ/n_p) is $U \approx 10^{-3.0 \pm 0.5}$ in both components. From the photoionization model, this corresponds to a neutral hydrogen fraction of $(H I)/H \approx 10^{-2.3 \pm 0.5}$. The abundances of the two components, given in Table 1, are both low and they differ. This is not a surprise because at early times, and in the outer regions of galaxies, there would be a large dispersion in abundances in gas which had not mixed. In both cases $[C/Si] \approx -0.3$, as is seen in Galactic stars which have similarly low metal abundances.

The low metallicity of this absorption system and standard models of chemical evolution^{9,10,16} imply negligible destruction

TABLE 1 Parameters from two-component model

Ion or derived quantity	Parameter	Blue component	Red component	Total
H I	N	17.74 ± 0.09	17.50 ± 0.19	17.94 ± 0.05
	b	17.1 ± 0.7	22.2 ± 3.0	
D I	N	13.10 ± 0.08	12.86 (tied)	13.30 ± 0.04
	b	14.0 ± 1.0	16.7 (tied)	
Si II	N	11.76 ± 0.07	12.41 ± 0.02	12.50 ± 0.02
	b	5.4 ± 1.6	9.6 ± 0.8	
Si III	N	12.73 ± 0.20	13.20 ± 0.05	13.33 ± 0.04
	b	5.5 ± 1.6	14.3 ± 2.0	
Si IV	N	12.02 ± 0.12	13.03 ± 0.02	13.07 ± 0.02
	b	3.8 ± 1.7	10.6 ± 0.5	
C II	N	12.70 ± 0.08	13.27 ± 0.03	13.37 ± 0.02
	b	7.0 ± 1.2	10.0 ± 0.5	
C III	N	13.36 ± 0.09	13.82 ± 0.19	13.95 ± 0.15
	b	7.9 ± 1.6	12.3 ± 1.1	
C IV	N	12.22 ± 0.16	12.61 ± 0.11	12.76 ± 0.08
	b	13.1 ± 5.9	9.2 ± 2.2	
O I				$N < 12.6(2\sigma)$
T ($10^4 K$)		1.62 ± 0.09	2.36 ± 0.09	
b_{turb}		4.8 ± 0.8	8.4 ± 0.4	
[C/H]		-3.0 ± 0.3	-2.2 ± 0.3	
[Si/H]		-2.8 ± 0.2	-1.9 ± 0.2	
[C/Si]		-0.2	-0.3	

Results from the simultaneous fit of the two-component model of the absorption system at $z = 3.572$ towards quasar 1937-1009. Column densities N are in units of cm^{-2} , and velocity dispersions (b -values) are in units of km s^{-1} . The 1σ random errors represent deviations that change the χ^2 of fits by 1σ . The absorption line parameters were taken from Morton³². The rightmost column shows the sum of the column densities in both components, which are much better determined than the distribution of the gas between the components. Hence the errors on the sums are smaller than on the individual components, and the best determination of D/H is the total D I column divided by the total H I column in the two components. The abundances of C and Si are presented in logarithmic units relative to solar abundances; for example, $[C/H] = -3$ means 10^{-3} of the solar C abundance. The upper limit on O I ($1,302 \text{ \AA}$) gives $[O/H] < -1.5$, for $U = 10^{-3}$. One b -value for each element at each velocity component provides a good fit to all the metal lines ($\chi^2 = 10.6$ for 8 degrees of freedom), and especially to the important blue components. Although the red components of the saturated Si III and C III lines both have b -values slightly larger than the mean, this is probably not significant because we do not see a trend with ionization.

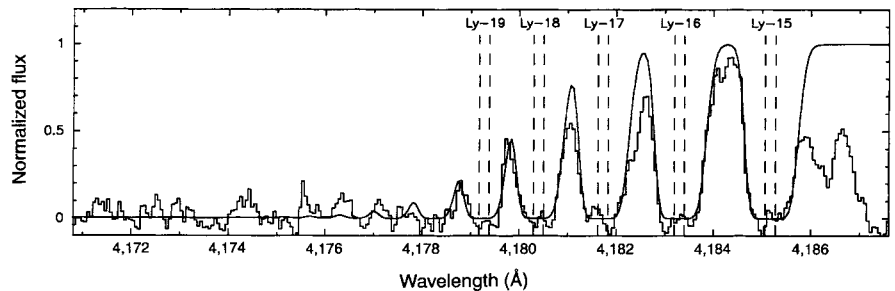
of deuterium from its primordial value, because stars which destroy D and eject H (without D) also eject metals such as Si.

We believe that our measured value of $D/H = (2.3 \pm 0.3 \pm 0.3) \times 10^{-5}$ is a detection, and not an upper limit, for the following reasons. First, the blue side of the Ly α line of the blue component is very steep, giving a $b(D)$ which is lower than 99% of H lines. Even if D/H in the two components is at the value found in the interstellar medium (ISM) and additional H at some nearby velocity accounts for most of this absorption, we find this contaminating H would have $b < 18 \text{ km s}^{-1}$, which is low enough to be unusual. Second, this $b(D)$ value agrees with the value predicted from the C II, Si II and H I lines. Third, our data on this absorption system are sensitive to D/H values of a few times 10^{-6} , because $N(H I)$ is large, the $b(H)$ is low, we see up to Lyman 19 and eight metal lines (which provide the redshifts, the component structure, b_{therm} and b_{turb}), the velocity structure is unusually simple and the signal-to-noise ratio is high. Fourth, we have simulated the expected overestimation of D/H due to contaminating H I, and find that the expected D/H is virtually identical to the D/H directly measured. This last point applied only to this data, and to this quasar absorption systems, which is much better constrained than all other such systems including those with possible high D/H.

Data on other absorption systems^{7,8,17,18} are about 10 times less sensitive because of lower H I columns or lower-quality spectra, and probably would not show $D/H \ll 10^{-4}$. There exist spectra of 50-100 quasars which have the sensitivity to detect $D/H \approx 10^{-4}$,

FIG. 2 Lyman-limit portion of the spectrum of quasar 1937–1009. The histogram represents measured flux in each pixel, normalized to the continuum level, and wavelength calibrated to heliocentric vacuum values. The smooth curve shows the Voigt profiles characterized by the values in Table 1.

METHODS. The spectra were obtained in five exposures covering 3890–7,450 Å on 31 July and 1 August 1994. We used a Textronix $2,303 \times 2,048$ CCD (charge-coupled device) with 2×2 pixel on-chip binning. A 1.14-arcsec slit was used to give a resolution of $\sim 8 \text{ km s}^{-1}$ measured from unblended lines in calibration spectra. All exposures covered 5,045–6,300 Å, which includes Ly α , and we added overlapping spectra, weighting each pixel by the square of its signal-to-noise ratio. Each exposure of quasar 1937–1009 was accompanied by dark, quartz lamp, and thorium–argon arc-lamp exposures. A standard star was also observed before or after exposures to trace the echelle orders and remove the blaze response in the spectra. The one-dimensional spectra were extracted from the two-dimensional images using an automated echelle extraction routine which performs baseline subtraction,



tion, bias subtraction, and flat-field correction before extracting the orders. The program also masks regions of the CCD subject to defects, and pixels struck by cosmic rays, and automatically finds wavelengths. The continuum level to the red of Lyman- α emission came from a sixth-order cubic spline with 3σ high and low pixel rejection. Between Lyman- α emission (5,820 Å) and the Lyman continuum break (4,170 Å), we used a third-, fourth-, or fifth-order Legendre polynomial, depending on the severity of the Lyman- α forest absorption, and we rejected pixels outside the range -1.2 to $+3\sigma$.

and perhaps one in 10 quasars has an absorption system which appears suitable, so we expect a few false coincidences where an H line lies at the expected position of D. These cases will give high upper limits on D/H because the $N(\text{H})$ values are too low to give the sensitivity needed to see low D/H values. Although the spectra of Q0014 + 813 (refs 7,8) are compatible with our D/H if their D line is H, our spectrum is completely incompatible with a high D/H value.

In the local interstellar medium, $\text{D}/\text{H} = (1.6 \pm 0.1) \times 10^{-5}$ (refs 6,19). If our measured value is the primordial value, then $\sim 30\%$ of D has been destroyed, and $\sim 30\%$ of atoms in the ISM have been inside stars, consistent with conventional models of Galactic chemical evolution²⁰. Edmunds⁹ has shown that the maximum depletion of D which can occur for arbitrary inflow and outflow of gas from the interstellar medium is $X_{\text{D}}/X_{\text{D}0} = f^{-1+1/\alpha}$, where $X_{\text{D}0}$ is the primordial mass fraction of D, X_{D} is the current mass fraction of D, f is gas mass divided by the total mass in gas plus stars (about 0.1–0.2 in the solar neighbourhood), and α is the fraction of mass formed into stars which remains in stellar remnants (conventionally taken as about 0.7–0.8). We then expect $X_{\text{D}}/X_{\text{D}0} \approx 0.5$ –0.7, in excellent agreement with our measurement of about 0.7. The best estimate of D/H in the gas from which the Sun formed is $(2.7 \pm 1.0 \text{ random} \pm$

$0.5 \text{ systematic}) \times 10^{-5}$ (ref. 21). The errors allow this value to lie between the current ISM value and our measurement, as it should.

Our D/H measurement implies a cosmological baryon-to-photon ratio of $\log \eta = -9.18 \pm 0.4 \pm 0.4 \pm 0.2$ (where the third error is the 1σ theoretical uncertainty from BBN predictions²) and a current density of baryons in terms of the critical density of $\Omega_{\text{b}} = 0.024^{+0.006}_{-0.005} h^{-2}$, where $H_0 = 100h \text{ km s}^{-1} \text{ Mpc}^{-1}$. Because the observed density of baryons in stars and hot gas is²² ~ 0.003 , most baryons ($\sim 94\%$ for $h = 0.7$) are dark matter, perhaps MACHOS in galaxy haloes²³, and the hot intergalactic gas which has now been seen²⁴ but not quantified.

Our D/H measurement implies a ^4He primordial mass fraction of $Y_{\text{p}} = 0.249 \pm 0.001 \pm 0.001 \pm 0.001$ (refs 3,4), which is higher than recent values from extragalactic H II regions; $Y_{\text{p}} = 0.228 \pm 0.005$ (ref. 25) and $Y_{\text{p}} = 0.241 \pm 0.003$ (ref. 26). But it is now realized that systematic uncertainties might allow $Y_{\text{p}} = 0.25$ (refs 27, 28). Our measurement also implies $\log(^7\text{Li}/\text{H}) = -9.3 \pm 0.1 \pm 0.1 \pm 0.15$, which is ~ 3 times the abundance seen in population II stars. Rotationally induced mixing (ref. 29) and stellar winds (ref. 30) can account for both the loss of ^7Li in these stars, and the variety of $^7\text{Li}/\text{H}$ amongst stars with similar temperatures and iron abundances (ref. 31). $\square$

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- Walker, T. P., Steigman, G., Schramm, D. N., Olive, K. A. & Kang, H. S. *Astrophys. J.* **376**, 51–69 (1991).
- Smith, M. S., Kawano, L. H. & Malaney, R. A. *Astrophys. J. Suppl. Ser.* **85**, 219–247 (1993).
- Krauss, L. M. & Kernan, P. J. *Phys. Lett. B* **347**, 347–353 (1995).
- Sarkar, S. *Rep. Prog. Phys.* (submitted).
- Copi, C. J., Schramm, D. N. & Turner, M. S. *Science* **267**, 192–199 (1995).
- Linsky, J. L. et al. *Astrophys. J.* **451**, 335–351 (1995).
- Songaila, A., Cowie, L. L., Hogan, C. J. & Rugers, M. *Nature* **368**, 599–603 (1994).
- Carswell, R. F., Rauch, M., Weymann, R. J., Cooke, A. J. & Webb, J. K. *Mon. Not. R. astr. Soc.* **268**, L1–L4 (1994).
- Edmunds, M. G. *Mon. Not. R. astr. Soc.* **270**, L37–L41 (1994).
- Steigman, G. & Tosi, M. *Astrophys. J.* **453**, 173–177 (1995).
- Petitjean, P. & Bergeron, J. *Astr. Astrophys.* **231**, 309–326 (1990).
- Vogt, S. S. et al. *Proc. SPIE* **2198**, 362–375 (1994).
- Hu, E. M., Kim, T. S., Cowie, L. L. & Songaila, A. *Astr. J.* **110**, 1526–1543 (1995).
- Donahue, M. & Shull, J. M. *Astrophys. J.* **383**, 511–523 (1991).
- Mathews, W. D. & Ferland, G. *Astrophys. J.* **323**, 456–467 (1987).
- Steigman, G. & Tosi, M. *Astrophys. J.* **401**, 150–156 (1992).
- Wampler, E. J. et al. *Astr. Astrophys.* (submitted).
- Carswell, R. F. et al. *Mon. Not. R. astr. Soc.* **278**, 506–518 (1996).
- McCullough, P. R. *Astrophys. J.* **390**, 213–218 (1992).
- Galli, D., Palla, F., Ferrini, F. & Penco, U. *Astrophys. J.* **443**, 536–550 (1995).
- Geiss, J. in *Origin and Evolution of the Elements* (eds Prantzos, N., Vangioni-Flam, E. & Cassé, M.) **89** (Cambridge Univ. Press, 1994).

- Persic, M. & Salucci, P. *Mon. Not. R. astr. Soc.* **258**, 14–18 (1992).
- Pratt, M. et al. (*MACHO collaboration*) *Workshop on Dark Matter in the Universe* Santa Monica, February (1996).
- Jakobsen, P. et al. *Nature* **370**, 35–39 (1994).
- Pagel, B. E. J., Simonson, E. A., Terlevich, R. J. & Edmunds, M. G. *Mon. Not. R. astr. Soc.* **255**, 325–345 (1992).
- Thuan, T. X., Izotov, Y. I. & Lipovetsky, V. A. in *Interplay between Massive Star formation, the ISM and Galaxy Evolution* (eds Kunth, D., Guiderdoni, B., Heydari-Malayeri, M. & Thuan, T. X.) (Editions Frontières Gif-sur-Yvette, 1996).
- Sasselov, D. & Goldwirth, D. *Astrophys. J.* **444**, L5–L8 (1995).
- Pagel, B. E. J. in *ESO/EIPC Workshop on Light Elements* (ed. Crane, P.) 155–164 (Springer, Berlin, 1994).
- Vauclair, S. & Charbonnel, C. *Astr. Astrophys.* **295**, 715–724 (1995).
- Charbonnel, C. *Astrophys. J.* **453**, L41–L44 (1995).
- Ryan, S. G., Beers, T. C., Deliyannis, C. P. & Thorburn, J. A. *Astrophys. J.* **458**, 543–560 (1996).
- Morton, D. C. *Astrophys. J. Suppl. Ser.* **77**, 119–202 (1991).
- Levshakov, S. A. & Kegel, W. H. *Mon. Not. R. astr. Soc.* **278**, 497–505 (1996).
- Gail, H. P., Hundt, E., Kegel, W. H., Schmid-Burgk, J. & Traving, G. *Astr. Astrophys.* **32**, 65–72 (1974).

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Abundance of ^3He in the local interstellar cloud

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The primordial abundances of the light elements and their isotopes provide essential information regarding the nucleosynthetic processes that occurred in the Big Bang^{1,2}. At present the best estimates of the baryon/photon ratio of the Universe, a fundamental cosmological parameter, are extrapolations to primordial times of light-element abundances measured in Solar System objects and in the Milky Way. Here we report a direct measurement of the $^3\text{He}/^4\text{He}$ abundance ratio in the local interstellar cloud, based on the isotopic analysis of helium atoms that have entered the Solar System from the surrounding interstellar medium. Our observations, together with the local-cloud D/H ratio³⁻⁵ and Solar System light-element data², permit an improved estimate of the baryon/photon ratio. We find that, within present limits of error, the sum of the ^3He and deuterium abundances has remained essentially unchanged since the formation of the Solar System, placing new constraints on models of Galactic chemical evolution⁶.

Interstellar helium atoms freely enter the Solar System with speeds of $\sim 25 \text{ km s}^{-1}$ due to the relative motion of the Sun with respect to the local interstellar cloud⁷. Influenced only by solar gravity, they penetrate deep into the heliosphere, where some become singly ionized, primarily by photoionization, and are swept outwards by the expanding solar wind⁸. Because all known physical processes that transform interstellar helium into the ionized helium found well inside the heliosphere (so-called pickup helium) are believed to affect ^3He and ^4He in similar fashion, the ratio of pickup $^3\text{He}^+/^4\text{He}^+$ measured close to the Sun represents the local interstellar cloud $^3\text{He}/^4\text{He}$ abundance.

Our observations of interstellar helium were made with the Solar Wind Ion Composition Spectrometer (SWICS)⁹ on the spacecraft Ulysses during a ~ 40 month period (July 1992 to November 1995). Long integration times were required because of the extremely low fluxes of ^3He pickup ions, and only periods of high solar-wind speed ($> 750 \text{ km s}^{-1}$) were included in order to maximize the detection efficiency of $^3\text{He}^+$. SWICS is well suited to identify and detect the rare, single-charged pickup ions^{10,11}, and separate them from the far more abundant, multiply-charged solar wind by using the triple coincidence time-of-flight versus energy technique which gives both a mass and charge determination and greatly reduces background¹².

The velocity distributions of solar-wind ions differ significantly from those of pickup ions. Solar-wind ions (and He^+ formed by recombination of solar-wind He^{2+} in charge-exchange collisions with interstellar atoms in the heliosphere) have roughly Maxwellian distributions with maximum intensity at the solar-wind speed, V_{sw} . Pickup ions have flat velocity distributions with a sharp cutoff at twice the solar-wind speed¹⁰. Beyond this cutoff the abundance of pickup ions is drastically reduced, except during times of large solar-wind turbulence generally associated with co-rotating and travelling shock waves when both solar-wind and pickup ions are accelerated¹³. Time intervals of strong turbulence were excluded from this analysis. The separation of pickup $^3\text{He}^+$ from $^4\text{He}^+$ and the far more abundant solar-wind ions was most effective at ion speeds between ~ 1.6 and 2 times V_{sw} as illustrated in Fig. 1 which shows clearly the presence of $^3\text{He}^+$.

The interstellar atomic number density of ^3He is equal to $F_{^3\text{He}^+}(W)/\int_{\Omega_{\text{inst}}} d\Omega f_{^3\text{He}^+}(w, \theta, \phi)$, with $F_{^3\text{He}^+} = C_{\text{inst}} \{r_3(W)/\xi_3(W)\} W^{-4}$ the measured velocity distribution of pickup $^3\text{He}^+$. The integration is over the instrument view angles, and $f_{^3\text{He}^+}$ is the

derived phase space density¹⁴ that includes all known processes affecting the transport of the ^3He gas from the local interstellar cloud to the location of Ulysses, and the production of pickup $^3\text{He}^+$ from atomic ^3He . C_{inst} is the instrument factor (a product of the geometry factor, energy/charge bandwidth and conversion factors), $r_3(W)$ the count rate of $^3\text{He}^+$ at W , the ion speed divided by V_{sw} , and $\xi_3(W)$ the triple coincidence efficiency. $f_{^3\text{He}^+}(w, \theta, \phi) = w^{-3/2} \{ \beta_{\text{prod}}/R \} N(w, R, \Theta, \beta_{\text{loss}}/V_o) G(\lambda_{^3\text{He}^+}, \theta, \phi)$, with β_{prod} the rate at which pickup $^3\text{He}^+$ is produced (primarily by photoionization of ^3He atoms) and R the heliocentric distance. $\mathbf{w} = \mathbf{W} - \mathbf{V}_{\text{sw}}/|\mathbf{V}_{\text{sw}}|$, and $\mathbf{V}_{\text{sw}}$ is the solar-wind flow vector. N is the normalized (to 1 in the local interstellar cloud, LIC) spatial distribution of neutral ^3He in the heliosphere as a function of spacecraft position coordinates R and Θ , and the ionization loss rate of helium atoms, β_{loss} , divided by V_o , the relative speed of the interstellar gas and the Sun. The function G describes the anisotropy of the pickup ion velocity distribution function in terms of $\lambda_{^3\text{He}^+}$, the pitch-angle scattering mean free path, and the direction angles θ and ϕ of the magnetic field relative to $\mathbf{V}_{\text{sw}}$. As identical expressions also hold for pickup $^4\text{He}^+$ (by replacing the

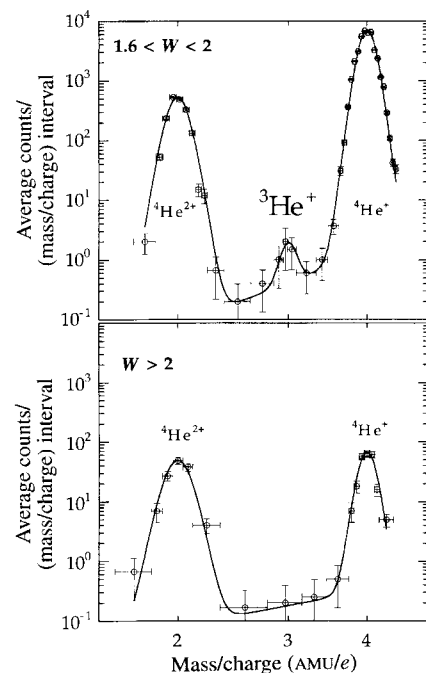


FIG. 1 Plot of the average triple coincidence counts of ions with masses between 2 and 6 AMU per mass per charge (m/q) intervals versus the mean m/q of each interval. Triple coincidence techniques used in SWICS enable us to identify each ion unambiguously by determining both its mass and mass/charge ratio. The top and bottom panels show ions with $1.6 < W < 2$, and $W > 2$ respectively, where W is the ion speed divided by the simultaneously measured solar-wind speed (both in the spacecraft frame of reference). The m/q intervals are unequal because in regions of low count rate the intervals were increased to contain at least one count. Use of the triple coincidence technique limits the residual background to very low levels, $\sim 10\%$ of the $^3\text{He}^+$ peak, and negligible for other ions shown. The peak around mass/charge of 3 for $1.6 < W < 2$ (top panel) is due to pickup $^3\text{He}^+$ (see text). No $^3\text{He}^+$ is expected, and none is observed for $W > 2$ (bottom panel). Note that $^3\text{He}^+$ is well resolved from both pickup $^4\text{He}^+$ and the suprathermal tail of solar-wind $^4\text{He}^{2+}$. The time intervals between the detection of adjacent $^3\text{He}^+$ ions followed a Poisson distribution with a mean of 121 days. The upper limit of that part of $^3\text{He}^+/^4\text{He}^+$ due to recombination of solar-wind $^3\text{He}^{2+}$ (a product of the counts ratio $^4\text{He}^{2+}/^4\text{He}^+$ for $1.6 < W < 2$ (top panel), the solar-wind $^3\text{He}^{2+}/^4\text{He}^{2+}$ ratio³⁵⁻³⁷, and the flux ratio¹⁰ of pickup hydrogen to solar-wind protons) is estimated to be $< 10^{-9}$. It is therefore completely negligible compared to the detected interstellar $^3\text{He}^+/^4\text{He}^+$.

index 3 with 4), the density ratio becomes $\{F_{3\text{He}^-}(W)/F_{4\text{He}^-}(W)\}/\{\int_{\Omega_{\text{int}}} d\Omega f_{3\text{He}^-}(w, \theta, \phi)/\int_{\Omega_{\text{int}}} d\Omega f_{4\text{He}^-}(w, \theta, \phi)\}$. The two integrals virtually cancel because $f_{3\text{He}^+}$ and $f_{4\text{He}^+}$ are nearly identical, and the interstellar $^3\text{He}/^4\text{He} \approx F_{3\text{He}^+}(W)/F_{4\text{He}^+}(W) = \{r_3(W)/r_4(W)\} \{\xi_4(W)/\xi_3(W)\}$. The triple coincidence efficiency ratio as a function of W is well known from pre-flight calibrations and its average is 1.4 ± 0.1 . We emphasize that all instrument-dependent uncertainties cancel, and that the error in determining $^3\text{He}/^4\text{He}$ is due primarily to counting statistics of $^3\text{He}^+$ ions.

To show that the derived phase space densities $f_{3\text{He}^-}$ and $f_{4\text{He}^+}$ are similar we note that the spatial distribution of neutral helium in the heliosphere, N , is determined primarily by the ionization loss rate of helium atoms. The loss rates are the same for ^3He and ^4He as ionization is a function of relative speed (for charge exchange) and atomic number, and not the mass of the atom. Screening of interstellar atoms by resonant charge-exchange processes beyond the termination shock^{15–18} will not affect either ^3He or ^4He . Just like the loss rate, the production rate of $^3\text{He}^+$ is equal to that of $^4\text{He}^+$. The anisotropy of the pickup ion distribution, G , depends on λ , the mean free path for scattering of low-energy ions in the solar wind. Pitch-angle scattering is found to be about the same for pickup ions as for electrons of comparable rigidity (momentum/charge ratio)¹⁴; the mean free path of such electrons shows no pronounced rigidity or mass dependence¹⁹. We estimate that a weak rigidity dependence will introduce a systemic error of at most $\pm 5\%$.

The measured velocity distribution for $^3\text{He}^+$ is compared to that of $^4\text{He}^+$ in Fig. 2. The $^3\text{He}^+/^4\text{He}^+$ ratio derived by least-squares (χ^2) fit from the ratio of these velocity distributions is 2.24×10^{-4} . The statistical 1σ upper and lower error limits, obtained from $\chi^2_{\text{min}} + 1$, are $+30\%$ and -26% respectively. The combined systemic error, resulting from uncertainties in the residual back-

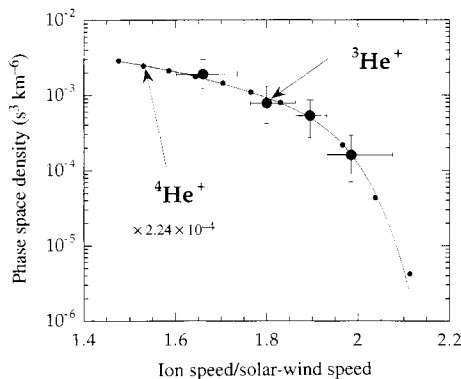


FIG. 2 Measured distribution functions, $F_m(W)$, of interstellar pickup $^3\text{He}^+$ and $^4\text{He}^+ \times 2.24 \times 10^{-4}$ versus the normalized ion speed, W . The phase space densities of $^3\text{He}^+$ ($m = 3$) and $^4\text{He}^+$ ($m = 4$) were computed from the count rates, $r_m(W)$, with $^3\text{He}^+$ corrected for a 10% background, and their respective efficiencies, $\xi_m(W)$. $F_m(W) = 4.541(r_m/\xi_m)(438/V_{\text{sw}})^4 W^{-4}$ (where V_{sw} is the solar-wind speed). Despite relatively large statistical uncertainties in the four values of the $^3\text{He}^+$ phase space densities, the similarity of the two spectral shapes is striking. No $^3\text{He}^+$ was observed above $W \approx 2$, consistent with the expected cutoff seen in the distribution functions of pickup ions^{10,38}. Below $W \approx 1.5$, the probability for triple coincidence becomes extremely small. SWICS measures the intensity of ions as a function of energy per charge (E), mass and charge state, from 0.6 to 60 keV e^{-1} in 64 logarithmically spaced steps with $\Delta E/E \approx 0.04$. Energy-per-charge analysis, followed by post-acceleration by 23 kV, a time-of-flight determination and energy measurement with solid-state detectors is used to identify ions and sample their distribution functions once every 13 minutes. At solar-wind speeds $> 750 \text{ km s}^{-1}$ the energy of pickup $^3\text{He}^+$ with speeds $> 1.6 \times V_{\text{sw}}$ was typically above the $\sim 40 \text{ keV}$ threshold of the solid-state detectors, and the probability of obtaining triple coincidence analysis was thus high. Only triple coincidence data were used to obtain the velocity distribution functions of interstellar $^3\text{He}^+$ and $^4\text{He}^+$.

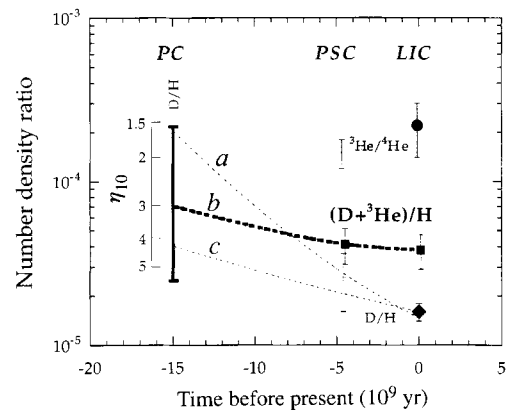


FIG. 3 Number density ratios of $^3\text{He}/^4\text{He}$, D/H and $(D + ^3\text{He})/H$ in three reservoirs separated in time. The Big Bang was assumed to have occurred at $-15 \times 10^9 \text{ yr}$. We consider each reservoir in turn. (1) Local interstellar cloud, LIC: $^3\text{He}/^4\text{He}$ is our present result, D/H was derived from Lyman- α absorption line measurements towards Capella and Procyon^{3–5}, and $(D + ^3\text{He})/H$ is the sum the D/H ratio and our $^3\text{He}/^4\text{He}$ value multiplied by $^4\text{He}/H = 0.10$. (2) Protosolar cloud, PSC^{2,23}: as virtually all of the protosolar D was converted to ^3He by the Sun, we use the well established present-day solar-wind $^3\text{He}/^4\text{He}$ ratio^{2,35–37}, multiplied by $^4\text{He}/H = 0.10$, for $(D + ^3\text{He})/H$, which is equal to $(4.1 \pm 1.0) \times 10^{-5}$. For the protosolar $^3\text{He}/^4\text{He}$ we take the meteoritic ratio²⁴ as is usually done, recognizing, however, that the circumstances of the incorporation of this helium component into the meteorites are not well known. D/H is simply the difference between solar-wind and meteoritic values multiplied by the protosolar $^4\text{He}/H$ ratio of ~ 0.1 . The derived D/H ratio depends critically on the assumption that the meteoritic $^3\text{He}/^4\text{He}$ correspond to the average PSC value. (3) Primordial clouds, PC: the range of D/H values shown have been obtained from measurements of highly red-shifted Lyman- α absorption lines of D and H towards distant quasars^{30–34}. Curves *a* and *b* bracket possible evolution of D/H . Curve *a* connects the LIC and PSC data with the high PC ratio³⁰ of $\sim 2 \times 10^{-4}$. Curves *b* and *c* represent typical galactic evolution²² of D/H and of $(D + ^3\text{He})/H$, leading to primordial ratios of $\sim 4 \times 10^{-5}$, and 7×10^{-5} , respectively. The baryon/photon ($\times 10^{10}$), η_{10} , scale corresponds to primordial D/H ; somewhat larger values of η_{10} should be used for primordial $(D + ^3\text{He})/H$.

ground, the efficiency ratios and anisotropies caused by differences in scattering mean free paths, is estimated to be $\sim 10\%$. The $^3\text{He}/^4\text{He}$ number density ratio in the local interstellar gas is thus found to be $\{2.2_{-0.6}^{+0.7}(\text{stat.}) \pm 0.2(\text{sys.})\} \times 10^{-4}$, where stat. indicates statistical error, and sys. indicates systematic error.

Using the 3.46-cm hyperfine line of $^3\text{He}^+$, measurements of $^3\text{He}/H$ in H II regions give widely different values in different locations of the interstellar medium^{20,21}, with a mean of $(2.42 \pm 1.45) \times 10^{-5}$. Although it is not clear how well this mean H II value represents the average interstellar medium abundance, it is entirely consistent with our LIC ratio (assuming $^4\text{He}/H \approx 0.1$), supporting the view that the LIC value is not anomalous but rather close to the average galactic abundance.

Figure 3 summarizes the present status of observations and interpretations of the light-isotope abundances in three reservoirs: the local interstellar cloud (LIC), the protosolar cloud (PSC) and the primordial clouds (PC), each representing a different epoch. The most noteworthy result derived from our measurement is that the value of $(D + ^3\text{He})/H$ is about the same in the LIC and PSC. The simplest inference that we can draw from this is that $(D + ^3\text{He})/H$ has not changed much during the $4.6 \times 10^9 \text{ yr}$ separating the PSC and LIC epochs, and that both the protosolar and present clouds have a composition that is typical for the Galaxy at the solar distance from the Galactic Centre. The absence of a significant secular change in $(D + ^3\text{He})/H$, compatible with some galactic evolution models²², confirms that this sum is particularly suitable for deriving its primordial value²³.

The modest increase of ^3He since the PSC epoch indicated in Fig. 3 was apparently compensated by a comparable decrease in D, presumably by conversion to ^3He . A monotonic decrease in D/H since primordial times is indeed expected because destruction of D processed in stars outweighs any known means of its production. The decrease in D/H since protosolar times corresponds to models with some infall (influx) of primordial gas²², assuming that the meteoritic value of $^3\text{He}/^4\text{He}$ (ref. 24) is indeed the PSC value. The chemical galactic evolution of ^3He is far more complicated because it is both produced and destroyed by stellar processing, and its secular trend is therefore much more difficult to predict^{22,25–27}. Some models indicate a much larger $^3\text{He}/\text{H}$ abundance in the PSC and present epochs, leading to suggestions²⁵ of observational bias in measurements of $^3\text{He}^+$ in HII regions; others²⁶ predict a decrease of ^3He due to enhanced stellar mixing. The observed small increase in the $^3\text{He}/^4\text{He}$ ratio since PSC times seems to be less than predicted by typical galactic evolution models²², indicating that ^3He production by low-mass stars may be lower than normally expected^{26,27}.

Both D and ^3He were produced in the Big Bang with abundances that were inversely related to the baryon-to-photon density ratio, η , in the present Universe^{28,29}, a fundamental cosmological invariant. Establishing the abundance of these isotopes limits possible values of η , a constant equally fundamental for the theory of matter and cosmology and one that provides a measure of the excess of matter over antimatter resulting from processes in the very early Universe. As is indicated in Fig. 3, a range of values of η of between 1.5×10^{-10} and 4×10^{-10} is compatible with presently observed abundances of the light-element isotopes.

Recently, deuterium has been discovered in primordial clouds using highly red-shifted Lyman- α absorption lines towards quasars^{30–34} yielding a range of D/H ratios up to $\sim 2 \times 10^{-4}$. A value of 2×10^{-4} is about ten times higher than D/H in the LIC, and corresponds to a primordial²⁹ (D + ^3He)/H ratio of 2.4×10^{-4} , six times higher than the measured LIC ratio. Most galactic evolution models²² predict considerably smaller differences in D/H and (D + ^3He)/H between primordial and present time. D/H ratios obtained from red-shifted Lyman spectroscopy vary considerably, making it difficult to derive a definitive primordial ratio at present. Further work on the spectra as well as on the nature and nuclear history of these red-shifted clouds may reveal the true primordial D/H ratio, and would thus provide a reliable value of η . With an accurate primordial D/H ratio, the protosolar cloud and local interstellar cloud light-element data would then, for the first time, place stringent and direct constraints on galactic evolution of deuterium and ^3He since primordial times. $\square$

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- Schramm, D. N. in *Origin and Evolution of the Elements* (eds Prantzos, N., Vangioni-Flam, E. & Cassé, M.) 112–131 (Cambridge Univ. Press, 1993).
- Geiss, J. in *Origin and Evolution of the Elements* (eds Prantzos, N., Vangioni-Flam, E. & Cassé, M.) 89–106 (Cambridge Univ. Press, 1993).
- Linsky, J. L. et al. *Astrophys. J.* **402**, 694–709 (1993).
- Linsky, J. L. et al. *Astrophys. J.* **451**, 335–351 (1995).
- Linsky, J. L. & Wood, B. E. *Astrophys. J.* (in the press).
- Steigman, G. & Tosi, M. *Astrophys. J.* **401**, 150–156 (1992).
- Witte, M., Rosenbauer, H., Banaskiewicz, M. & Fahr, H. J. *Adv. Space Res.* **13**, 121–126 (1993).
- Holzer, T. E. *Rev. Geophys. Space Phys.* **15**, 467–490 (1977).
- Gloeckler, G. et al. *Astr. Astrophys. Suppl. Ser.* **92**, 267–289 (1992).
- Gloeckler, G. et al. *Science* **261**, 70–73 (1993).
- Geiss, J. et al. *Astr. Astrophys.* **282**, 924–933 (1994).
- Gloeckler, G. *Rev. Sci. Instrum.* **61**, 23613–23620 (1990).
- Gloeckler, G. et al. *J. Geophys. Res.* **99**, 17637–17643 (1994).
- Gloeckler, G., Schwadron, N. A., Fisk, L. A. & Geiss, J. *Geophys. Res. Lett.* **22**, 2665–2668 (1995).
- Baranov, V. B. & Malama, Yu. G. *J. geophys. Res.* **98**, 15157–15163 (1993).
- Baranov, V. B. & Malama, Y. G. *J. geophys. Res.* **100**, 755–761 (1995).
- Ripken, H. W. & Fahr, H. J. *Astr. Astrophys.* **122**, 181–192 (1983).
- Zank, G. P., Pauls, H. L., Williams, L. L. & Hall, D. T. in *Proc. Solar Wind 8 Conf.* (eds Winterhalter, D. & Neugebauer, M.) (Am. Geophys. Union, in the press).
- Palmer, I. D. *Rev. Geophys. Space Phys.* **20**, 335–349 (1982).
- Rood, R. T., Bania, T. M., Wilson, T. L. & Balser, D. S. in *ESO/EIPC Workshop on the Light Elements* (ed. Crane, P.) 201–214 (Springer, Heidelberg, 1995).
- Balser, D. S., Bania, T. M., Brockway, C. J., Rood, R. T. & Wilson, T. L. *Astrophys. J.* **430**, 667–681 (1994).

- Tosi, M. in *From Stars to Galaxies* (eds Leitherer, C., Fritze-von Alvensleben, C. & Huchra, J.) (Conf. Ser., Publ. Astr. Soc. Pacific, in the press).
- Geiss, J. & Reeves, H. *Astr. Astrophys.* **18**, 126–132 (1972).
- Eberhardt, P. *Earth planet. Sci. Lett.* **24**, 182–187 (1974).
- Olive, K. A., Rood, R. T., Schramm, D. N., Truran, J. & Vangioni-Flam, E. *Astrophys. J.* **444**, 680–685 (1995).
- Wasserberg, G. J., Boothroyd, A. I. & Sackmann, I.-J. *Astrophys. J.* **447**, L37–L40 (1995).
- Prantzos, N. *Astr. Astrophys.* (in the press).
- Wagoner, R. V., Fowler, W. A. & Hoyle, F. *Astrophys. J.* **148**, 3–49 (1967).
- Walker, T. P., Steigman, G., Schramm, D. N., Olive, K. A. & Kang, H.-S. *Astrophys. J.* **376**, 51–69 (1991).
- Songaila, A., Cowie, L. L., Hogan, C. J. & Rugers, M. *Nature* **368**, 599–604 (1994).
- Rugers, M. & Hogan, C. J. *Astrophys. J.* **459**, L1–L5 (1996).
- Carswell, R. F. et al. *Mon. Not. R. astr. Soc.* **268**, L1–L4 (1994).
- Tytler, D. & Fan, X. M. *Nature* **381**, 207–209 (1996).
- Tytler, D. in *QSO Absorption Lines* (ed. Meylan, G.) (Springer, in the press).
- Bodmer, R., Bochsler, P., Geiss, J., von Steiger, R. & Gloeckler, G. *Space Sci. Rev.* **72**, 61–64 (1995).
- Möbius, E. et al. *Nature* **318**, 426–429 (1985).
- Geiss, J., Bühler, F., Cerutti, H., Eberhardt, P. & Filleux, C. in *Apollo 16 Preliminary Science Report Ch. 14* (SP-315, NASA, Washington DC 1972).
- Coplan, M. A., Oglvie, K. W., Bochsler, P. & Geiss, J. *Solar Phys.* **93**, 415–434 (1984).

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Optical patterning of multi-domain liquid-crystal displays with wide viewing angles

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THE leading technology for flat, high-resolution computer and television screens is based on twisted nematic liquid-crystal displays¹. The successful operation of these displays requires control of molecular alignment, which is currently achieved by confining the liquid crystal between mechanically rubbed surfaces². But in addition to the practical difficulties associated with rubbing, the resulting displays suffer from restricted viewing angles arising from the uniaxial nature of the alignment process^{3,4}. This latter problem can in principle be circumvented if molecular alignment is varied, in a controlled manner, within individual pixels^{5–9}. Exposure of functionalized substrates to polarized light offers a means of achieving high-resolution patterns in the plane of the display^{10–12}. But to ensure that the alignment pattern imposed on the liquid crystal is free of orientation defects, the tilt angle between the long molecular axes and the substrates must be precisely controlled^{13,14}. Here we show how our earlier linear photoalignment strategy^{10,12} can be extended to obtain such control, and thereby fabricate stable, multi-domain pixel displays with markedly improved fields of view.

Underlying the electro-optical properties of all liquid-crystal displays is the requirement that, in their 'off' state (no applied electric field), the long axes of the liquid-crystal molecules—the director $\hat{n}$ —are aligned uniaxially at the two display boundaries. Moreover, to achieve stable electro-optical characteristics, the bias tilt angle θ between $\hat{n}$ and the display substrate(s) must also be well defined. Previously we have shown that anisotropic Van der Waals forces are primarily responsible for the alignment of liquid crystals on substrates treated with our linear photopolymerization (LPP) technology^{10,12}. In the case of poly(vinyl 4-methoxycinnamate) photopolymers, these forces were shown to align $\hat{n}$ perpendicular to the electric field vector $\hat{E}$ of the incident linearly polarized ultraviolet radiation which leads to anisotropic depletion of LPP prepolymer molecules and simultaneously generates uniaxially aligned photoproducts via anisotropic crosslinking^{10,12}.

Because of the cylinder symmetry with respect to $\hat{\mathbf{E}}$ of this LPP process, the probabilities are equal for photogenerating the bias tilt angles $\theta(x) = -\theta(-x)$ on a microscopic scale, where x, y are the substrate coordinates. Macroscopically this leads to zero bias tilt¹⁰. Although attempts were made to break the symmetry of our LPP process by exposing LPP substrates sequentially under different directions of light incidence, the resulting angles $\theta < 0.3^\circ$ were very small⁸ and not stable for practical purposes¹².

From these symmetry considerations it follows that the photo-crosslinking and aligning symmetries of the LPP process had to be changed. We achieved this with a modified LPP process schematically depicted in Fig. 1, such that liquid-crystal alignment occurs not perpendicular to $\hat{\mathbf{E}}$ as before^{10,12} but within the plane defined by $\hat{\mathbf{E}}$ and $\hat{\mathbf{k}}$ of the incident crosslinking ultraviolet radiation. As a consequence of the new symmetry and by changing the angle of incidence of $\hat{\mathbf{k}}$, stable, photoinduced bias tilt angles θ have become possible which can be adjusted over the entire range $\theta = 0-90^\circ$. As an example, the top part of Fig. 1 schematically depicts two coumarin photo-prepolymer molecules which we designed to exhibit the desired LPP-aligning symmetry. Also shown in Fig. 1 (centre) are two of the four possible in-plane configurations of the anisotropically aligned coumarin photoproducts generated by the (2 + 2)-cycloaddition of the LPP process. Analogous to our earlier LPP model^{10,12} we assume that liquid-crystal alignment $\hat{\mathbf{n}}$ in the y -direction occurs (1) via anisotropic

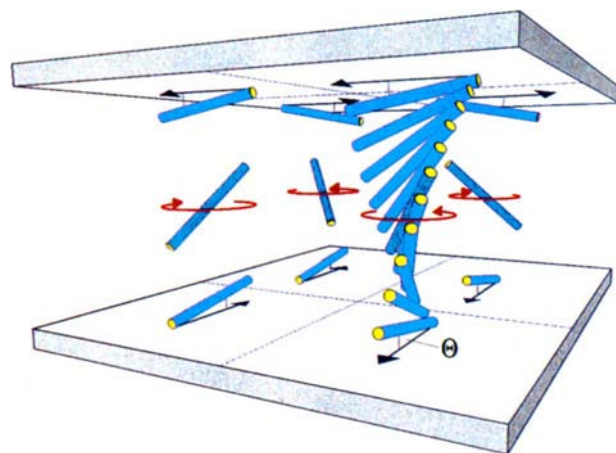


FIG. 2 A single pixel of a four-domain twisted nematic (TN) LCD configuration in its partly switched-on state (80% transmission between crossed polarizers). Three of the four (differently twisted) subtwisted nematic helices are indicated by their respective boundary and central molecules.

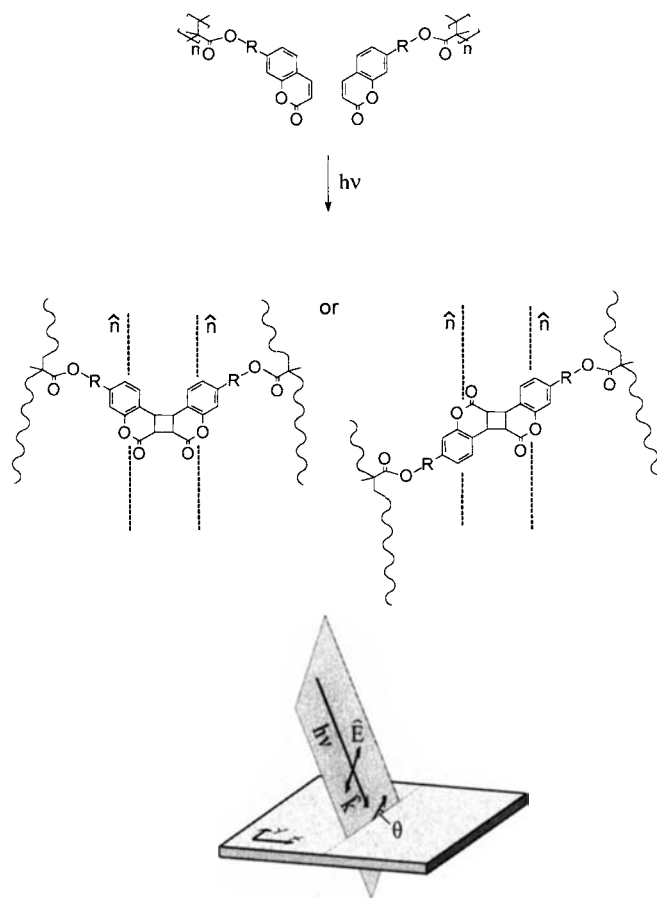


FIG. 1 A pair of isotropically distributed, novel prepolymer coumarin side-chain molecules, for use in the LPP process. The directional LPP photoreaction is parallel to the direction of the electric field vector $\hat{\mathbf{E}}$ of the incident linearly polarized ultraviolet radiation; R , side-chain spacer. The photoinduced bias tilt angles $\hat{\mathbf{n}}(\theta)$ are adjustable from $\theta = 0-90^\circ$. The photoalignment is optically and thermally stable up to temperatures $> 200^\circ\text{C}$. The diagram at the bottom of the figure schematically depicts the photo-generation and the symmetry of our LPP process.

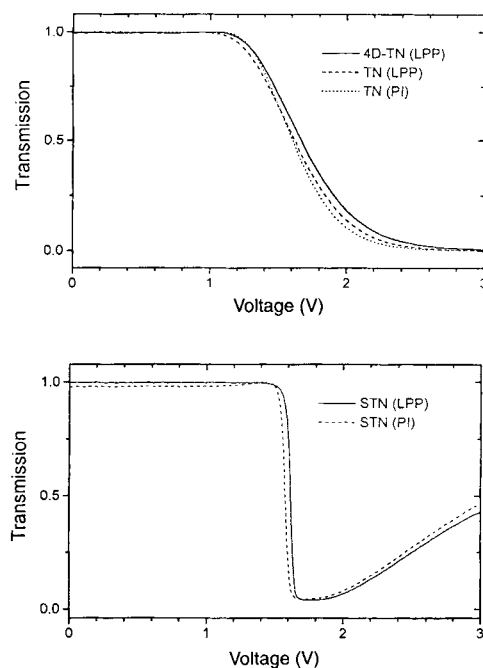


FIG. 3 Top, transmission-voltage characteristics at vertical white-light incidence of a photoaligned, single-domain twisted nematic (TN) display (dashed line) and a four-domain TN display with 100- μm subpixel size (solid line). Both LPP-aligned displays exhibit $\theta(\text{LPP}) = 1.3^\circ$ bias tilt. For comparison (dotted line) we show characteristics of the polyimide (PI)-brushed display with $\theta(\text{PI}) = 1.5^\circ$ bias tilt; PI SE510 from Nissan Chemical. The three displays are operated in their first minimum, and use TFT mixture 8457 from ROLIC Ltd. Bottom, transmission-voltage characteristics of a single-domain photoaligned supertwisted nematic (STN) display (solid line) and of a conventionally, PI-brushed STN display (dashed line), both with contrast ratios > 25 . The respective slope steepnesses are $V_{90}/V_{10} = 1.043$ (LPP) and 1.046 (PI); where 90 and 10 correspond to the respective display transmission in per cent. The respective bias tilt angles are $\theta(\text{LPP}) = 7.5^\circ$ and $\theta(\text{PI}) = 7.0^\circ$; twist angle $\phi = 240^\circ$; STN mixture 7728 from ROLIC Ltd; PI SE610 from Nissan Chemical.

Van der Waals interactions of the rigid cores of the anisotropically crosslinked photoproducts, (2) via anisotropic steric interactions with their partly aligned (in the y -direction) hydrocarbon polymer chains and (3) via anisotropically depleted prepolymer molecules (not shown in Fig. 1). All the photoaligned displays below are based on the new LPP symmetry shown in Fig. 1. The LPP photoprocessing steps have been described elsewhere^{10,12}.

Figure 2 depicts schematically, a partially switched single pixel of a four-domain twisted nematic (TN) display configuration. As in the photoaligning patterning process described recently¹⁵, we used a single photomask to generate the LPP-aligning patterns of Fig. 2. The two different bias tilt directions θ on each substrate determine the appropriate twist sense of the four subtwisted nematic liquid-crystal displays (LCDs). Different bias tilt angles were achieved by changing the direction of the wavevector $\hat{k}$ (Fig. 1). On a macroscopic scale, the optical anisotropies of the differently aligned central directors $\hat{n}$ of the four sub-helices compensate each other (Fig. 2). Therefore, the angular dependence of four-domain displays is strongly reduced compared with single-domain displays. In Fig. 2 this is indicated by the emphasised central molecules of the four differently aligned, single-domain sub-helices.

We show in Fig. 3, top panel, the vertical transmission-voltage characteristics of two LPP-photoaligned TN displays. For com-

parison, a polyimide (PI)-brushed, conventional single-domain TN display is included. One of the photo-aligned displays is single-domain, the other is a photopatterned four-domain display according to Fig. 2. From Fig. 3 and the large contrast ratios > 150 of all three displays, it follows that the static transmission-voltage characteristics at vertical light incidence of photoaligned single- and multi-domain displays are identical to those of conventional, brushed displays. This also holds for the dynamics of the three displays.

The large bias tilt angles, which are prerequisites for realizing stable highly twisted configurations exhibiting very steep transmission-voltage characteristics, could up to now only be achieved with brushed substrates. The only partially photoaligned super-twisted nematic (STN) display which we have recently described was made by combining a (low-tilt) photoaligned substrate with a high-tilt brushed substrate^{10,12}. As a consequence it was difficult to reach the steep transmission-voltage characteristics required for realizing multiplexed high-information-content STN displays. Figure 3, lower panel, shows the stable, hysteresis-free transmission-voltage characteristics of the first, fully photoaligned STN display at vertical white-light incidence. For comparison an identically configured display with brushed PI-substrates is shown. The equally steep slopes of the two graphs in Fig. 3 and the comparably large bias tilt angles demonstrate that photo-

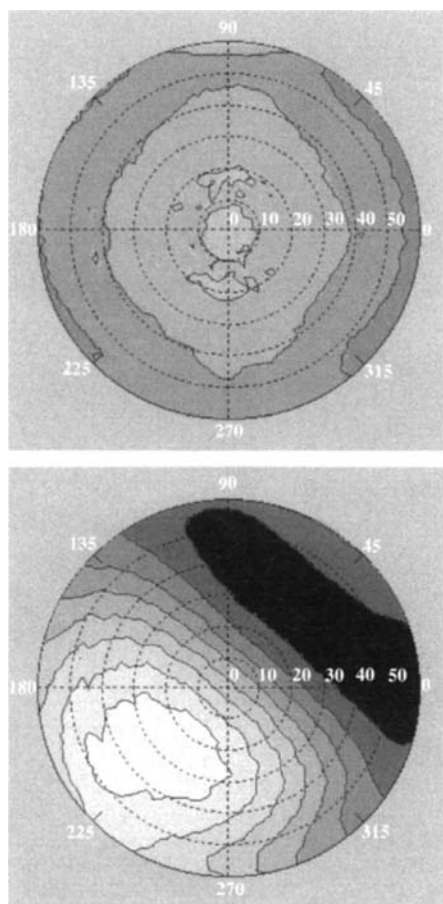


FIG. 4 Viewing-angle dependence of the brightness at 50% vertical transmission of LPP-aligned four-domain TN-LCD (top) and PI-aligned single-domain TN-LCD (bottom). The angular dependence of the LPP-aligned single-domain display of Fig. 3 is identical to that of the PI-aligned single-domain display. The intensity shadings which are separated by azimuthal angular contours correspond to the (ten) levels of intensity transmitted by the displays at different angles of light incidence in the four quadrants. The maximum display transmission range is divided into ten intensity levels; black corresponds to the minimum display transmission.

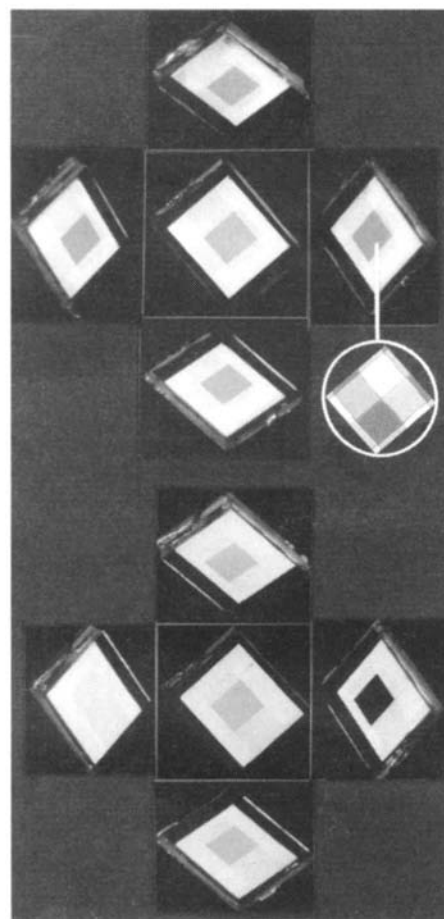


FIG. 5 Viewing-angle dependence of an LPP-aligned four-domain TN display (top) and of a PI-aligned single-domain display (bottom). The pixel area of both displays is 1 cm^2 . The subpixel size of the four-domain display is $100 \mu\text{m}$. At vertical incidence (central pictures) both displays exhibit identical grey levels corresponding to 70% transmission in Fig. 3. (See text for discussion of ringed, higher-magnification, picture.) The four pictures around each central picture show the displays when viewed at 40° (with respect to the retical) in each of the four quadrants.

aligned, highly multiplexable and high-contrast supertwisted displays have become feasible.

Figure 4 demonstrates the remarkably improved field of view of a photo-aligned four-domain TN displays (top) compared with the strongly angle-dependent brightness of a conventionally brushed, single-domain display (bottom). The angular dependence of the brightness of the two partially switched-on displays in Fig. 4 was measured in all four quadrants between vertical light incidence and 60° off-axis. The grey levels at vertical light incidence are identical for both displays, and correspond to 50% transmission in Fig. 3. Figure 4 shows that, instead of remaining constant, the brightness of the conventional display changes by nine grey levels (out of ten) within its first and third quadrant, whereas the brightness of the photoaligned four-domain display changes by only three grey levels within any of its four quadrants.

The remarkably improved viewing performance of photo-aligned multi-domain displays is further illustrated by the photographs in Fig. 5 which show the angular dependence of the grey level of a large single pixel at 70% vertical transmission of an LPP-aligned four-domain display (top) and of a PI-aligned single-domain display (bottom). The centre pictures were taken at vertical light incidence, the others at 40° in the four quadrants. The comparison is self explanatory. Also shown in Fig. 5 (ringed) is an enlarged view of a four-domain display pixel viewed at 40° in the first quadrant. This shows the four photopatterned and differently aligned TN-subpixels whose macroscopically averaged transmission accounts for the improved angular view of the photoaligned four-domain display.

We have shown that high-resolution (< 5 µm) liquid-crystal

aligning patterns with defined and broadly adjustable bias tilt angles have become feasible with our linear photoalignment technology. For this reason, and because of its stability with respect to light and heat, photoalignment renders mechanical alignment with its detrimental generation of dust particles and electrostatic charges obsolete. LPP photoalignment of monomeric and polymeric liquid crystals not only improves the performance of existing displays, such as their viewing properties, but also opens up interesting new display configurations and non-display-related optically anisotropic devices¹⁵, such as optically patternable interference filters, polarizers and optical retarders. □

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- Schadt, M. & Helfrich, W. *J. Appl. Phys. Lett.* **18**, 127–128 (1971).
- Cognard, J. *Molec. Cryst. Liq. Cryst. Suppl. Ser.* **1**, 1–74 (1982).
- Meyerhofer, D. *J. Appl. Phys.* **48**, 1179–1185 (1977).
- Luo, F. C. in *Liquid Crystals* (ed Bahadur, B.) Vol. 1 (World Scientific, Singapore, 1990).
- Yang, K. H. *Jap. J. appl. Phys. Lett.* **31**, 1603–1605 (1992).
- Sumiyoshi, K., Takatori, K., Hirai, Y. & Kaneko, S. *J. Soc. for Information Display* **2/1** 31–33 (1994).
- Kawata, Y., Takato, K., Hasegawa, M. & Sakamoto, M. *Liq. Cryst.* **16**, 1027–1036 (1994).
- Hashimoto, T. et al. *Digest SID '95* 877–880 (Soc. for Information Display, Playa del Rey, CA, 1995).
- Chen, J. et al. *J. Appl. Phys. Lett.* **67**, 1990–1992 (1995).
- Schadt, M., Schmitt, K., Kozinkov, V. & Chigrinov, V. *Jap. J. appl. Phys.* **31**, 2155–2164 (1992).
- Gibbons, W. M., Shannon, P. J., Sun, S. T. & Swetlin, B. J. *Nature* **351**, 49–50 (1991).
- Schadt, M., Seiberle, H., Schuster, A. & Kelly, S. M. *Jap. J. appl. Phys.* **34**, 3240–3249 (1995).
- Raynes, E. P. *Electron. Lett.* **10**, 141–142 (1974).
- Scheffer, T. J. & Nehring, J. *J. Appl. Phys. Lett.* **45**, 1021–1023 (1984).
- Schadt, M., Seiberle, H., Schuster, A. & Kelly, S. M. *Jap. J. appl. Phys. Lett.* **34**, 764–767 (1995).

Detection of nonlinear dynamics in short, noisy time series

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THE accurate identification of deterministic dynamics in an experimentally obtained time series^{1–5} can lead to new insights regarding underlying physical processes, or enable prediction, at least on short timescales. But deterministic chaos arising from a nonlinear dynamical system can easily be mistaken for random noise^{6–8}. Available methods to distinguish deterministic chaos from noise can be quite effective, but their performance depends on the availability of long data sets, and is severely degraded by measurement noise. Moreover, such methods are often incapable of detecting chaos in the presence of strong periodicity, which tends to hide underlying fractal structures⁹. Here we present a computational procedure, based on a comparison of the prediction power of linear and nonlinear models of the Volterra–Wiener form¹⁰, which is capable of robust and highly sensitive statistical detection of deterministic dynamics, including chaotic dynamics, in experimental time series. This method is superior to other techniques^{1–6,11,12} when applied to short time series, either continuous or discrete, even when heavily contaminated with noise, or in the presence of strong periodicity.

Consider the usual description of a dynamical system as a 'black box' with input x_n and output y_n , at time $n = 1, \dots, N$ in multiples of the sampling time τ . The discrete Volterra series is then a Taylor-like polynomial expansion of y_n in terms of

$x_n, x_{n-1}, \dots, x_{n-\kappa+1}$, where κ is the memory of the system. To overcome the computational intractability of the Volterra series, Wiener introduced an orthogonal formulation¹³ assuming that x_n is a gaussian white noise sequence over an infinite time period. Korenberg's recasting of the expansion¹⁴, on which our approach is based, relaxes these restrictions thereby allowing its application to finite and arbitrary signals.

For a dynamical system, either strictly autonomous or reformulated as such, we propose a closed-loop version of the Volterra series in which the output y_n feeds back as delayed input (that is, $x_n \equiv y_{n-1}$). Within this framework, we analyse univariate time series by using a discrete Volterra–Wiener–Korenberg series of degree d and memory κ as a model to calculate the predicted time series y_n^{calc} :

$$y_n^{\text{calc}} = a_0 + a_1 y_{n-1} + a_2 y_{n-2} + \dots + a_\kappa y_{n-\kappa} + a_{\kappa+1} y_{n-1}^2 + a_{\kappa+2} y_{n-1} y_{n-2} + \dots + a_{M-1} y_{n-\kappa}^d = \sum_{m=0}^{M-1} a_m z_m(n) \quad (1)$$

where the functional basis $\{z_m(n)\}$ is composed of all the distinct combinations of the embedding space coordinates¹⁵ ($y_{n-1}, y_{n-2}, \dots, y_{n-\kappa}$) up to degree d , with a total dimension $M = (\kappa + d)!/(d!\kappa!)$. Thus, each model is parametrized by κ and d , which correspond to the embedding dimension and the degree of nonlinearity of the model, respectively. The coefficients a_m are recursively estimated through a Gram–Schmidt procedure¹⁴ from linear and nonlinear autocorrelations of the data series itself. The calculations can be readily performed on a workstation.

The short-term prediction power of a model is then measured by the standard one-step-ahead prediction error

$$\varepsilon(\kappa, d)^2 \equiv \frac{\sum_{n=1}^N (y_n^{\text{calc}}(\kappa, d) - y_n)^2}{\sum_{n=1}^N (y_n - \bar{y})^2} \quad (2)$$

where $\bar{y} = 1/N \sum_{n=1}^N y_n$ and $\varepsilon(\kappa, d)^2$ is in effect a normalized variance of the error residuals. We now search for the best

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model $\{\kappa_{\text{opt}}, d_{\text{opt}}\}$ that minimizes the following information criterion¹⁶ in accordance with the parsimony principle:

$$C(r) = \log \varepsilon(r) + r/N \quad (3)$$

where $r \in [1, M]$ is the number of polynomial terms of the truncated Volterra expansions from a certain pair $\{\kappa, d\}$.

The numerical procedure is as follows: for each data series, obtain the best linear model by searching for κ^{lin} which minimizes $C(r)$ with $d = 1$. Repeat with increasing κ and $d > 1$, to obtain the best nonlinear model. Likewise, obtain the best linear and nonlinear models for surrogate randomized data sets with the same autocorrelation (and power spectrum) as the original series^{3,5}. This results in four competing models with error standard deviations $\varepsilon_{\text{orig}}^{\text{lin}}, \varepsilon_{\text{orig}}^{\text{nl}}, \varepsilon_{\text{surr}}^{\text{lin}}$ and $\varepsilon_{\text{surr}}^{\text{nl}}$.

From above, the presence of nonlinear determinism is indicated if $d_{\text{opt}} > 1$. Further corroboration is obtained with the following objective statistical criteria: for models with gaussian residuals, a standard F -test will serve to reject, with a certain level of confidence, the hypothesis that nonlinear models are no better than linear models as one-step-ahead predictors. This gaussian assumption was verified throughout our analysis by using a χ^2 -test with a 99% cutoff. Alternatively, the results are confirmed by using the nonparametric Mann–Whitney rank-sum statistic, which does not depend on the gaussian assumption³. Under this scheme, the relevance of nonlinear predictors is established when the best nonlinear model from the original data is significantly more predictive than both (1) the best linear model from the data series, and (2) the best linear and nonlinear models obtained from the surrogate series, that is, $\varepsilon_{\text{orig}}^{\text{lin}}, \varepsilon_{\text{surr}}^{\text{nl}}, \varepsilon_{\text{surr}}^{\text{lin}} > \varepsilon_{\text{orig}}^{\text{nl}}$ in the statistical sense.

Two points are noteworthy. First, because surrogate data are generated by preserving only the linear autocorrelation function of the data series (nonlinear autocorrelations are randomized),

the addition of nonlinear terms does not increase the prediction power. As expected, $\varepsilon_{\text{surr}}^{\text{nl}} \approx \varepsilon_{\text{surr}}^{\text{lin}}$ and surrogate data are always best approximated by a linear model.

Second, when dealing with continuous signals, the time delay τ for the embedding (or optimal sampling rate) is another free parameter to be determined¹⁷. For detection purposes, the optimal time delay τ_{opt} may be chosen so as to maximize the difference between $\varepsilon_{\text{orig}}^{\text{lin}}$ and $\varepsilon_{\text{orig}}^{\text{nl}}$. The value of τ_{opt} is bounded by two limits. On one hand, if $\tau \gg \tau_{\text{opt}}$ (undersampling) all four models (linear and nonlinear, original or surrogate) will have similarly small prediction powers and nonlinearity cannot be detected. On the other hand, for an oversampled data series with a small step size ($\tau \ll \tau_{\text{opt}}$), the linear correlation of the embedding is so large that linear models always prevail. Within the range of acceptable time delays where τ_{opt} lies, generally $\varepsilon_{\text{orig}}^{\text{lin}} \approx \varepsilon_{\text{surr}}^{\text{lin}}$; that is, when optimally sampled, the prediction power of the linear model of a continuous signal derives mainly from its autocorrelation function. This equivalence of linear models from original and surrogate data holds for discrete maps as well. Consequently, and in contrast with other methods, surrogate data play only a confirmatory role in our procedure.

We have tested our algorithm with a wide variety of short time series, typically 1,000 points long. As a preliminary trial, we verified the method with several linear signals which, in some cases, proved to be non-recognizable by some of the existing methods (see discussions in refs 3 and 8). The examples included: white gaussian and coloured $1/f^2$ noises⁷; an autoregressive linear random process³; periodic sinusoidal and nonsinusoidal series (see Theiler in ref. 8); as well as a quasiperiodic signal from a torus with two frequencies (Paluš in ref. 8). In all cases the method yielded $\varepsilon_{\text{lin}} \leq \varepsilon_{\text{nl}}$ and the linear hypothesis could not be rejected. The same conclusion was reached in the presence of noise or when an *a posteriori* nonlinear transformation was performed on the original linear data⁵.

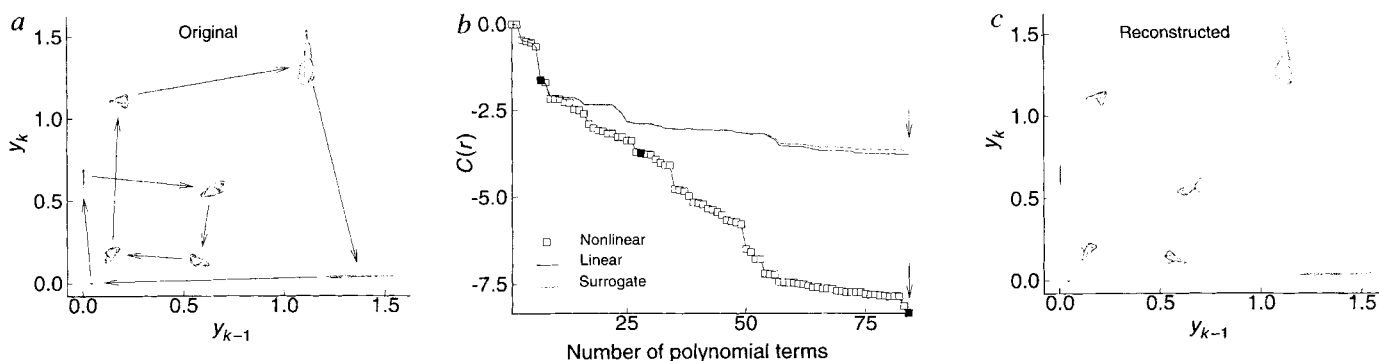


FIG. 1 a, Embedding reconstruction of an ecological model⁸: $x_k = \lambda y_{k-1} \exp(-0.001(y_{k-1} + x_{k-1}))$; $y_k = 0.2x_{k-1} \exp(-0.07(y_{k-1} + x_{k-1})) + 0.8y_{k-1} \exp(-0.05(y_{k-1} + 0.5x_{k-1}))$ with $\lambda = 118$. The trajectory evolves in an attractor comprised of fractals in several disconnected domains and visits each of them in a periodic manner as indicated by the arrows. This strong periodicity makes detection of the chaotic component difficult. b, $C(r)$ (see equation (3)); r is the number of polynomial terms) for a set of nonlinear, linear and surrogate data models for a time series (1,000 points) from the same ecological model, no noise added. Linear and surrogate models with $d = 1$ and differing κ (see text) are represented. The nonlinear models are complete (■) or truncated (□) Volterra series with fixed $\kappa = 6$ and varying d . Optimal nonlinear, linear and surrogate models are indicated by the arrows. Here $F_{\text{calc}} = (\varepsilon_{\text{orig}}^{\text{nl}})^2 / (\varepsilon_{\text{orig}}^{\text{lin}})^2 = 9,387 \gg F(0.01, 915, 915) = 1.17$. Alternatively, the Mann–Whitney statistic is $Z = -37.13 \ll -2.33$, the value of the inverse normal cumulative density function for $\alpha = 0.01$. Similarly, the information criterion $C(r)$ indicates that $d_{\text{opt}} > 1$. From these three criteria we deduce the nonlinear component is significant. c, Attractor propagated from a model ($\kappa = 2, d = 8$) obtained with the algorithm. It captures the general shape and subfractal structure of the attractor of the original non-Volterra model. d, Plot of $1/F_{\text{calc}}$ versus the percentage of added coloured noise for the same ecological example. The noise percentage is defined as the ratio of the standard deviations of the additive random series and the original data. The discontinuous line indicates the 99% confidence interval. The nonlinear deterministic component can be reliably detected even in the presence of $\sim 50\%$ correlated noise. Similar results (not shown) are obtained for the connected fractal with $\lambda = 140$.

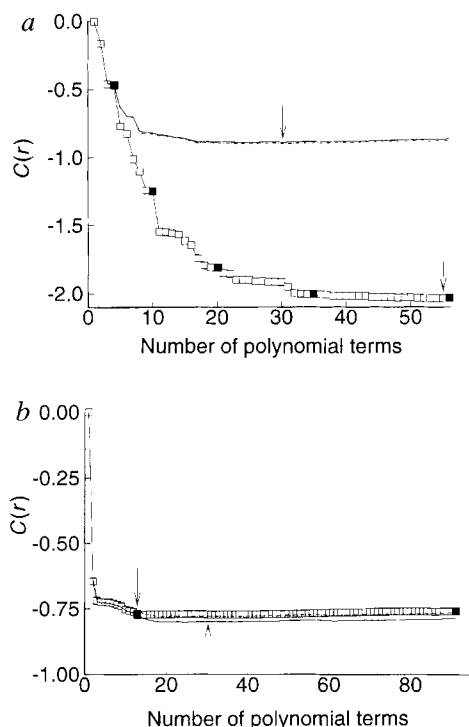


FIG. 2 *a*, As Fig. 1*b*, but for an experimental time series (1,000 points) of emission of an NH_3 laser from an apparatus designed to produce Lorenz-like chaos (series A from ref. 8). In this case $F_{\text{calc}} = 10.23 > F(0.01, 968, 944) = 1.16$, and the nonparametric Mann–Whitney statistic is $Z = -24.60 < -2.33$. Both criteria (and $C(r)$) indicated that the nonlinear component is significant. *b*, As *a*, but for an experimental time series (1,874 points) of the intensity of a variable dwarf star (segments 1 and 2 of series E from ref. 8) in which linear superposition of modes had been presumed and was to be verified. Not only is $d_{\text{opt}} = 1$ but the hypothesis of nonlinearity may be rejected with a 94% confidence level as $1/F_{\text{calc}} = 1.08 \approx F(0.06, 1855, 1783)$.

Table 1 summarizes some of the results for numerically generated nonlinear examples. It is important to emphasize the diversity of the data sets. For example, most of the discrete series (except the Hénon and logistic maps) have non-polynomial, non-Volterra functional forms. The detection algorithm worked equally well with continuous systems, including those that evolve around several ghost centres (Lorenz, Duffing); high-dimensional systems (as in series D (ref. 8), with a dimension of 9); and chaotic series from nonlinear delayed feedback mechanisms (Mackey–Glass equation) with implicit dimension of seven.

We also examined the robustness of our method in the presence of noise by adding to all the nonlinear examples white and/or coloured measurement noises—the latter with the same autocorrelation as the original series (Table 1). Even with such short series, nonlinearity was detected under high levels of noise (~ 40 –

TABLE 1 Results of algorithm tests

Discrete systems	%	Continuous systems	%
Logistic map	70	Rössler	75
Hénon map	70	Duffing	40
Ikeda map	65	Lorenz II (ref. 3)	50
Ecological model ⁹	50	Series D (ref. 8)	50
Non-chaotic fractal ²¹	25	Mackey–Glass ⁵	15

List of numerically generated nonlinear examples and approximate maximum percentages of correlated noise that can be added to a 1,000-points-long series before the nonlinear component is no longer detected with a 99% cutoff. These maximum values increase for longer data series.

75%) comparable to those achieved by other methods with much longer ($\sim 16,000$ points) series³.

As an example of the power of the algorithm, Fig. 1 shows results for an ecological model whose chaotic component, obscured by strong periodicity, has eluded detection by some of the existing methods even in the absence of noise⁹. In contrast, our technique—without any reprocessing of the data—detected the presence of nonlinearity even when the series was corrupted by $\sim 50\%$ additive correlated noise. Moreover, the algorithm is robust against contamination of the signal with random spikes (Poisson impulses) of intensities comparable to the signal amplitude and density up to 0.02.

Dynamic noise—random noise recycled by the dynamics of the system—is a major source of variability in feedback systems¹⁸. We addressed this issue by numerically introducing white and/or coloured dynamic noise in Hénon and Ikeda maps with boundary constraints to keep the trajectories in the attractors. Encouragingly, the method detected nonlinearity up to similar levels of noise as for the purely additive case, that is, $\sim 80\%$ and $\sim 70\%$ respectively.

Finally, we tested our algorithm with two benchmark experimental series (Fig. 2); the results are in agreement with published conclusions⁸.

Some questions remain concerning the significance of the models so obtained. Although the choice of model selection criterion is not critical for the present purposes, the reliability of the resulting models as long-term predictors (for example, Fig. 1*c*) is highly dependent on the chosen criterion, the nature of the time series and the amount of noise present (M.B. and C.-S.P., unpublished work). The procedure can be extended to non-polynomial functional bases¹⁹ or multivariate series. But these approaches may not be feasible for large-scale functional expansions unless efficient algorithms (such as the recursive one proposed here become available. New directions for the method, including iterative refinement of models with shadowing²⁰ or other noise-reduction techniques, may prove useful for modelling in the presence of noise and full detection of chaos. The demonstrated robustness of the technique in a wide variety of test examples suggests its possible applicability to general experimental data. □

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- Sugihara, G. & May, R. M. *Nature* **344**, 734–741 (1990).
- Casdagli, M. *Physica D* **35**, 335–356 (1989).
- Kennel, M. B. & Isabelle, S. *Phys. Rev. A* **46**, 3111–3118 (1992).
- Theiler, J., Eubank, S., Longtin, A., Galdrikian, B. & Farmer, J. D. *Physica D* **58**, 77–94 (1992).
- Kaplan, D. T. & Glass, L. *Physica D* **64**, 431–454 (1993).
- Grassberger, P. & Procaccia, I. *Physica D* **9**, 189–208 (1983).
- Osborne, A. R. & Provenzale, A. *Physica D* **35**, 357–381 (1989).
- Weigend, A. S. & Gershenfeld, N. A. (eds) *Time Series Prediction* (Santa Fe Inst. Studies in the Sciences of Complexity, Vol. XV, Addison-Wesley, Reading, MA, 1994).
- Cazelles, B. & Ferrière, R. H. *Nature* **355**, 25–26 (1992).
- Marmareis, V. Z. (ed.) *Advanced Methods of Physiological System Modeling* Vol. II (Plenum, New York, 1988).
- Grassberger, P. & Procaccia, I. *Phys. Rev. A* **28**, 2591–2593 (1983).

- Sano, M. & Sawada, Y. *Phys. Rev. Lett.* **55**, 1082–1085 (1985).
- Wiener, N. *Nonlinear Problems in Random Theory* (Wiley, New York, 1958).
- Korenberg, M. J. *Ann. biomed. Eng.* **16**, 123–142 (1988).
- Takens, F. in *Dynamical Systems and Turbulence* (eds Rand, D. A. & Young, L. S.) 336–381 (Lecture Notes in Mathematics Vol. 898, Springer, Berlin, 1981).
- Akaike, H. *IEEE Trans. Auto. Control* **19**, 716–723 (1974).
- Fraser, A. M. & Swinney, H. L. *Phys. Rev. A* **33**, 1134–1140 (1986).
- Glass, L. & Malta, C. P. *J. theor. Biol.* **145**, 217–223 (1990).
- Crutchfield, J. P. & MacNamara, B. S. *Complex Systems* **1**, 417–452 (1987).
- Hammel, S. M. *Phys. Lett. A* **148**, 421–428 (1990).
- Grebogi, C., Ott, E., Pelikan, S. & Yorke, J. A. *Physica D* **13**, 261–268 (1984).

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Global and hemispheric CO₂ sinks deduced from changes in atmospheric O₂ concentration

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The global budget for sources and sinks of anthropogenic CO₂ has been found to be out of balance unless the oceanic sink is supplemented by an additional 'missing sink', plausibly associated with land biota^{1,25}. A similar budgeting problem has been found for the Northern Hemisphere alone^{2,3}, suggesting that northern land biota may be the sought-after sink, although this interpretation is not unique²⁻⁵; to distinguish oceanic and land carbon uptake, the budgets rely variously, and controversially, on ocean models^{2,6,7}, ¹³CO₂/¹²CO₂ data^{2,4,5}, sparse oceanic observations of *p*CO₂ (ref. 3) or ¹³C/¹²C ratios of dissolved inorganic carbon^{4,5,8} or single-latitude trends in atmospheric O₂ as detected from changes in O₂/N₂ ratio^{9,10}. Here we present an extensive O₂/N₂ data set which shows simultaneous trends in O₂/N₂ in both northern and southern hemispheres and allows the O₂/N₂ gradient between the two hemispheres to be quantified. The data are consistent with a budget in which, for the 1991–94 period, the global oceans and the northern land biota each removed the equivalent of approximately 30% of fossil-fuel CO₂ emissions, while the tropical land biota as a whole were not a strong source or sink.

We focus on time series from Alert (82.5° N), La Jolla (32.9° N) and Cape Grim (40.7° S), as shown in Fig. 1. The data reveal seasonal cycles, interannual trends, and gradients with latitude. We express changes in O₂/N₂ relative to a reference according to

$$\delta(\text{O}_2/\text{N}_2) = (\text{O}_2/\text{N}_2)/(\text{O}_2/\text{N}_2)_{\text{ref}} - 1$$

where we multiply $\delta(\text{O}_2/\text{N}_2)$ by 10⁶ and express the results in units of 'per meg' (used here to express parts-per-million changes in a ratio). For each site we compute the annual means centred on 1 January and 1 July. We average together the annual means for La Jolla and Alert which we use as a proxy $\delta(\text{O}_2/\text{N}_2)_{\text{NH}}$ for the Northern Hemisphere as a whole, and we use the Cape Grim data as a proxy $\delta(\text{O}_2/\text{N}_2)_{\text{SH}}$ for the Southern Hemisphere as a whole. We then compute $\delta(\text{O}_2/\text{N}_2)_{\text{GLOBAL}} = (\delta(\text{O}_2/\text{N}_2)_{\text{NH}} + \delta(\text{O}_2/\text{N}_2)_{\text{SH}})/2$, which we use as a measure of the global mean, and $\delta(\text{O}_2/\text{N}_2)_{\text{GRAD}} = \delta(\text{O}_2/\text{N}_2)_{\text{NH}} - \delta(\text{O}_2/\text{N}_2)_{\text{SH}}$, which we use as a measure of the north-south gradient. We compute the same statistics for concurrent CO₂ data from the same flask samples.

Figure 2 shows that the temporal trends in (CO₂)_{GLOBAL} and $\delta(\text{O}_2/\text{N}_2)_{\text{GLOBAL}}$ are well correlated, with a period of anomalously slow growth in CO₂ concentration centred around 1992^{11,12} coinciding with a period of slower decrease in O₂/N₂. If fossil-

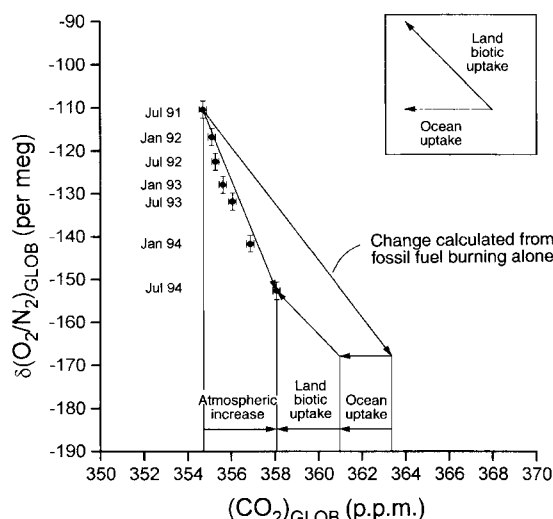
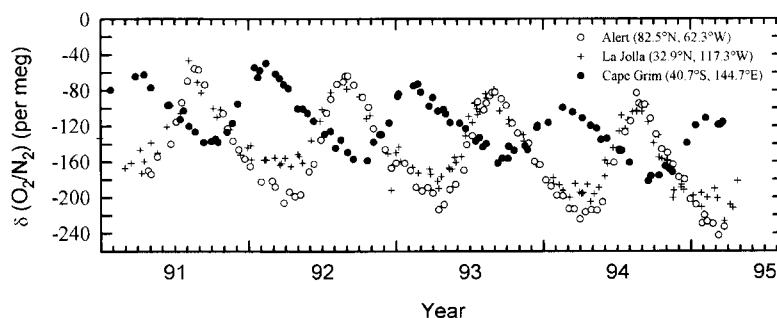


FIG. 2 Globally and annually averaged $\delta(\text{O}_2/\text{N}_2)$ versus CO₂. Annual means are computed by first fitting the data to the sum of a stiff spline plus a four-harmonic annual cycle. The harmonic fit is then used to adjust data to the 15th of each month, and monthly means are computed. Twelve consecutive monthly means are averaged to compute annual means. Months with missing data are filled in using the complete fitted curve. Also shown are the trends in CO₂ and O₂/N₂ computed from fossil-fuel burning and cement manufacture^{32,27}, and the unique combination of oceanic and land biotic CO₂ uptake required to account for the observations. Oceanic uptake of CO₂ is assumed to have no effect on O₂ concentration⁹, while land biotic uptake is assumed to occur with O₂/CO₂ ratios of -1.1:1 (ref. 33). The error bars indicate the estimated standard errors of the globally and annually averaged values. In computing global oceanic and land biotic uptake, we additionally allow for uncertainty of ± 6 per meg and ± 0.1 p.p.m. in the overall trends in O₂/N₂ and CO₂, respectively, due to uncertainty in long-term calibration.

fuel burning were the only process inducing changes, then atmospheric CO₂ and O₂/N₂ would have changed by +8.9 p.p.m. and -60.4 per meg, respectively, from 1991 to 1994. In comparison, the observed changes were 3.2 ± 0.26 p.p.m. and -42 ± 6 per meg. Assuming the N₂ concentration is constant, and that exchanges with land biota are the only additional long-term influence on O₂ levels, we can use the trend in O₂/N₂ to allocate CO₂ uptake between land biota and the oceans⁹. The system can be solved graphically, as shown in Fig. 2. We find that the land biosphere and the oceans removed 2.9 ± 1.3 and 2.4 ± 1.2 p.p.m., respectively, over the three-year period, corresponding to average annual sinks of 2.0 ± 0.9 and 1.7 ± 0.9 Pg C yr⁻¹, respectively (1 Pg = 10¹² kg). Similar analysis using La Jolla data alone to infer global trends yields a land biotic sink of 1.8 ± 0.7 Pg C yr⁻¹ and an oceanic sink of 1.9 ± 0.5 Pg C yr⁻¹ over the longer period from 1989 to 1994.

The north-south gradients in atmospheric CO₂ concentrations and O₂/N₂ ratio depend on the spatial and temporal pattern of CO₂ and O₂ sources and sinks as modified by atmospheric

FIG. 1 $\delta(\text{O}_2/\text{N}_2)$ of air samples collected at Alert, La Jolla and Cape Grim and measured using the interferometric method³⁴. Data from additional sites in the Scripps O₂/N₂ network (not shown) indicate that the interannual trends and north-south gradients inferred from these three sites are characteristic of large-scale atmospheric patterns.



mixing. The main sources and sinks believed to contribute to the gradients are fossil-fuel burning (which occurs mostly in the Northern Hemisphere), the natural pumping of CO₂ and O₂ from the northern to the southern hemisphere by the thermohaline circulation of the Atlantic Ocean, the exchange of CO₂ and O₂ with land biota, and the uptake of excess anthropogenic CO₂ by the oceans^{2,3,13–15}.

A plot of $\delta(\text{O}_2/\text{N}_2)_{\text{GRAD}}$ versus $(\text{CO}_2)_{\text{GRAD}}$ for data from 1991 to 1994 is shown in Fig. 3a. The data show clear interannual variations, with both gradients being largest (in absolute value) in 1991 and early 1992 and smallest in early 1993. The average CO₂ gradient is close to the 3 p.p.m. gradient observed during the middle 1980s. As seen in Fig. 3a, the time-averaged gradients, $\delta(\text{O}_2/\text{N}_2)_{\text{GRAD}}$ and $(\text{CO}_2)_{\text{GRAD}}$, are both smaller in absolute value than predicted from fossil-fuel burning alone using an atmospheric transport model¹⁶. In seeking an explanation for the reduction of both gradients, we note that CO₂ uptake by land biota in the Northern Hemisphere decreases both gradients, whereas transport by the North Atlantic thermohaline circulation decreases the CO₂ gradient while increasing the O₂/N₂ gradient. The Atlantic Ocean pumps O₂ (relative to N₂) in the same direction (north to south) as CO₂ because the flux of both gases is primarily caused by temperature-dependent solubility differences between northward- and southward-flowing waters^{15,17}. The relative oceanic transport of O₂ and CO₂ can be estimated from ocean tracer distributions¹⁵.

We find from model simulations similar to those in Table 1 that the gradients in O₂/N₂ ratio and CO₂ concentration can neither be simultaneously explained by combining fossil-fuel burning and land biotic exchanges alone, nor can they be explained by additionally including an Atlantic thermohaline component, if the latter is based on north-south transport as large as 0.6 Pg C yr⁻¹ (ref. 13) or larger². A consistent explanation of both gradients is possible, however. We illustrate this by starting with *a priori* estimates of the oceanic and fossil-fuel contributions to the north-south gradients plus an arbitrary land biotic contribution, and then adjusting these using an objective method¹⁸ to construct a consistent model, as summarized in Table 1 and Fig. 3b.

The consistent model is achieved without major adjustments in the oceanic components. The Atlantic thermohaline transport of 0.4 Pg C yr⁻¹ is partially offset by a component due to oceanic uptake of anthropogenic CO₂, which increases the CO₂ gradient but does not affect the O₂/N₂ gradient. The total oceanic contribution to the gradient is constrained to be quite small (-0.32 ± 0.43 p.p.m.), so that most of the reduction in CO₂ gradient must be explained by land biota. The total land-biotic component (-1.31 ± 0.87 p.p.m.) has a relatively large uncertainty, however, because it has an O₂/CO₂ signature similar to

TABLE 1 Components of O₂/N₂ and CO₂ north-south gradients

Parameter	(Units)	Symbol	<i>a priori</i>		<i>a posteriori</i>	
			Value	σ	Value	σ
Fossil-fuel CO ₂ grad.*	(p.p.m.)	C _f	4.91	0.69	4.90	0.68
Ocean CO ₂ uptake grad.†	(p.p.m.)	C _o	0.42	0.42	0.42	0.41
Land biotic CO ₂ grad.‡	(p.p.m.)	C _l	-1.15	5.0	-1.31	0.87
North Atlantic CO ₂ grad.§	(p.p.m.)	C _{na}	-0.78	0.35	-0.78	0.34
North Atlantic O ₂ /N ₂ grad.§	(per meg)	O _{na}	-4.0	1.9	-4.0	1.9
North Atlantic multiplier§		β_{na}	1.0	1.0	0.96	0.49
Fossil-fuel O ₂ /CO ₂ combust. ratio		α_f	1.38	0.04	1.38	0.04
Land biota O ₂ /CO ₂ exch. ratio¶		α_l	1.10	0.05	1.10	0.05
Observed CO ₂ grad.#	(p.p.m.)	C _{obs}	3.27	0.26	3.27	0.26
Observed O ₂ /N ₂ grad.#	(per meg)	O _{obs}	-29.2	2.4	-29.2	2.3
Combined results:						
North Atlantic CO ₂ gradient	(p.p.m.)	$\beta_{na}C_{na}$			-0.75	0.41
Total ocean CO ₂ gradient	(p.p.m.)	$\beta_{na}C_{na} + C_o$			-0.32	0.43

The '*a priori*' column contains estimates based on prior information, as described in the footnotes below. The '*a posteriori*' column contains the results of the objective optimization consistent with the governing equations which are: $C_{obs} = C_f + C_o + C_l + \beta_{na}C_{na}$ and $O_{obs} = -(\alpha_f C_f + \alpha_l C_l) / X + \beta_{na} O_{na}$ where $X = 0.2095$ is the O₂ mole fraction of dry air.

* The gradient induced by fossil-fuel burning in 1992. The gradients were computed using the TM2 atmospheric transport model¹⁶ with fossil-fuel production (6.1 Pg C yr⁻¹) and source distribution data from refs. 26,27. We allow for 10% uncertainty (1 σ) in fossil-fuel production and distribution, and 10% uncertainty in transport, which we sum in quadrature to yield a total uncertainty of 14%. The fossil-fuel gradients were corrected for fossil-fuel emissions of CO and CH₄ and corrected for photochemical oxidation of CO and CH₄ (whether from fossil fuels or other sources) using a photochemistry model²⁸.

† The gradient induced by the uptake of anthropogenic CO₂ by the oceans, estimated using a model estimate²⁹ of the CO₂ uptake pattern scaled to 1.7 Pg C yr⁻¹ and using the TM2 model. We allow an uncertainty of 100%.

‡ The CO₂ gradient induced by exchanges with land biota. This component includes the contributions from net exchanges plus the rectification of seasonal exchanges. We use essentially no *a priori* constraint here.

§ The gradients induced by pre-industrial transport of O₂ and CO₂ from the North Atlantic into the Southern Hemisphere by the Atlantic's thermohaline circulation. The *a priori* estimates use transports of $(0.36 \pm 0.17) \times 10^{14}$ mol O₂ and $(0.33 \pm 0.15) \times 10^{14}$ mol CO₂, as deduced from water-mass differences in O₂, total carbon, nitrate-corrected alkalinity, phosphate and salinity¹⁵. The O₂ flux here is corrected for the compensating flux of N₂, where the N₂ flux was assumed proportional to the heat flux (see also ref. 14). The *a priori* CO₂ transport adopted here is consistent with other recent estimates although uncertainties are large^{17,30,31}. We include an arbitrary scaling factor β_{na} to allow for additional uncertainty in the overall strength of the thermohaline circulation¹⁵. The CO₂ and O₂ fluxes were distributed according to the source/sink pattern for the 'North Atlantic sink' defined in ref. 20, and used as input to the TM2 model.

|| Based on fossil-fuel O₂ consumption factors for different fuel types³². We allocate O₂ consumption by country, allowing for differences in the mixture of fuels used by different countries. The global O₂/CO₂ ratio for fossil-fuel burning (plus cement manufacturing) for 1992 is 1.39 assuming complete combustion. The factor of 1.38 used here also allows for differences in the spatial pattern of O₂ consumption and CO₂ production as this affects ratios of the signals predicted at the three sampling sites using the TM2 model, it allows for incomplete combustion of fossil-fuel, and it allows for atmospheric photochemical oxidation of CH₄ and CO, as per the first footnote to this table.

¶ From ref. 33.

Computed by taking the mean and standard error of the gradients for the four calendar years 1991–94.

the much larger fossil-fuel component, and is thus affected by small percentage errors in the estimated production and atmospheric transport of fossil-fuel CO₂.

Land biota can influence the CO₂ and O₂/N₂ gradients both by net annual exchanges and also by purely seasonal exchanges that are rectified by seasonal variations in atmospheric mixing^{2,19}. Taking an estimate of $+0.62 \pm 0.33$ p.p.m. for the gradient due to rectification based on the range of two model predictions^{2,19} for the sites under consideration, we calculate, by difference, a residual component due to net exchanges (-1.93 ± 0.93 p.p.m.). The only plausible explanation for such a gradient is net exchange of CO₂ with land biota in the Northern Hemisphere, because tropical exchanges have minimal effect on the north-south gradient, and because there is relatively little extra-tropical land biota in the Southern Hemisphere. The gradient (-1.93 ± 0.93 p.p.m.) requires a northern sink of about 1.9 ± 0.9 Pg C yr⁻¹, assuming that the sink is proportional to net primary production on land between 39° and 63° N (ref. 20).

Thus we find that the net CO₂ uptake by land biota in the Northern Hemisphere which is needed by our analysis to explain the north-south gradients is similar in magnitude to the net uptake required at the global scale by the interannual trends in

O_2/N_2 ratio and CO_2 concentration. One implication is that tropical ecosystems were not a strong net source or sink for CO_2 over the 1991–94 period. If so, then any releases of CO_2 from tropical deforestation must have been offset by CO_2 uptake occurring elsewhere in the tropics, as indicated by measurements during 1992/93 in Amazonia²¹.

Our results are sensitive to the transport model due, in part, to the sparseness of the sampling network. Errors arising from such uncertainties in the fossil-fuel and rectifier terms have been explicitly considered. Our analysis does not, however, consider all possible sources of error. The estimated northern land biotic

sink assumes that the sink is widespread over middle latitudes; a sink of unknown distribution would have larger uncertainty. Also, although our data indicate the need for an oceanic contribution to the combined O_2/N_2 and CO_2 gradients, our attribution of this component to the Atlantic thermohaline circulation remains speculative. More work is needed to characterize better the patterns of O_2 and CO_2 exchange in the Atlantic and other oceans and to account for river fluxes.

Our analysis neglects possible gradients induced by the rectification of purely seasonal oceanic exchanges. Preliminary results from the Hamburg model of the ocean carbon cycle²² suggest the effect on $\delta(O_2/N_2)_{GRAD}$ is smaller than 1 per meg. An ocean rectifier effect of at least 4 per meg would be needed to significantly affect our results.

Another possible source of error is interannual variations in air–sea exchange of O_2 driven by imbalances in marine photosynthesis and ventilation rates. Indeed, we see evidence for such exchanges in our data. The ~ 14 per meg reduction in the O_2/N_2 gradient between January 1992 and January 1993 (Fig. 3a) was ~ 7 per meg too large relative to the 1.4 p.p.m. shift in CO_2 gradient if both shifts were caused solely by exchanges with land biota. We speculate that the ~ 7 per meg excess shift in O_2/N_2 gradient was produced by anomalous exchanges of O_2 with the Southern Ocean. Interestingly, the steepest O_2/N_2 gradient and the largest seasonal O_2/N_2 increase at Cape Grim (see Fig. 1 and ref. 10) both occurred in 1991/92. Both features could be explained by an anomalous pulse of $\sim 1 \times 10^{14}$ mol O_2 released from the Southern Ocean around January 1992. Allowing for an uncompensated oceanic O_2 pulse of this magnitude, our estimated global land biotic uptake decreases from 2.0 to 1.7 Pg Cyr^{-1} and our estimated northern land biotic uptake increases from 1.9 to 2.05 Pg Cyr^{-1} . This suggests that errors in our previous estimates due to such an event are relatively small. Nevertheless, the O_2/N_2 and CO_2 data alone are not sufficient in general to quantify interannual imbalances in O_2 or CO_2 driven by ocean biota^{10,23}. Our analysis assumes these exchanges are small; other data sets are needed to quantify such exchanges.

The growth rate of atmospheric CO_2 was unusually slow during the 1991–94 period compared to the previous decade, and particularly compared to the period of high growth rate in 1987 and 1988^{11,12}. The slowdown has been ascribed (based on $^{13}C/^{12}C$ data^{12,24}) to increasing CO_2 uptake by land biota, an interpretation which is compatible with our analysis for the 1991–94 period only if the northern land biota were a weaker sink or tropical land biota were a net CO_2 source in the 1980s. Our global oceanic sink is consistent with model estimates of long-term oceanic uptake that require net land biotic uptake averaging 0–1 Pg Cyr^{-1} to balance the carbon budget over the past 30 years (ref. 25).

Our results are in rough agreement with two recent studies, one by Bender *et al.*¹⁰, who found a global land biotic sink of 3.3 ± 1.6 Pg Cyr^{-1} for the 1992–93 period based on independent O_2/N_2 records from 40° S and 41° S, and the other by Ciais *et al.*⁷ who found a land biotic sink from 30° to 60° N of ~ 3.5 Pg Cyr^{-1} in the 1992–93 period based on the north–south gradient in CO_2 and $^{13}C/^{12}C$ isotopes. The Bender *et al.* records agree well with the Cape Grim record (Fig. 1) in the critical overlapping time periods, thus supporting our global flux estimates which are based on longer records from both hemispheres. As the Ciais *et al.* study was based on a more comprehensive observational dataset but from a slightly different time period and used a different transport model, more analysis is needed to assess if the O_2/N_2 and $^{13}C/^{12}C$ data are consistent with the same source/sink distributions. The O_2/N_2 data complement the stable-isotope data by providing additional constraints, without complications from isotope dilution effects, on the partitioning of the global CO_2 sink between the land and the oceans and among large-scale regional sources and sinks. □

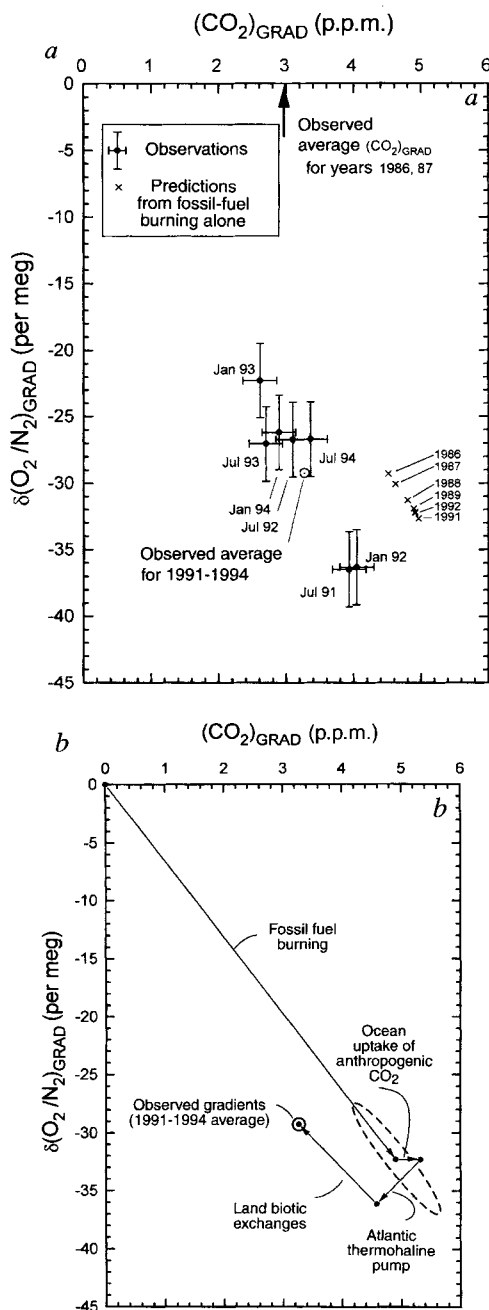


FIG. 3 a, Observed gradient components for 1991–94 plus modelled gradients from fossil-fuel burning (corrected for CO and CH_4 oxidation, see Table 1) plus cement manufacturing for years 1986 to 1992. Also shown are the average gradient $(CO_2)_{GRAD}$ for the years 1986 and 1987, computed using data from Cape Grim, Alert and Kumukahi³ after correcting for an average offset of 0.815 p.p.m. observed between Kumukahi and La Jolla⁶. b, The *a posteriori* components of the average $\delta(O_2/N_2)$ and CO_2 gradients as resolved by the objective optimization (Table 1). The ellipsoid shows the range allowed by uncertainty in fossil-fuel burning and transport only.

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1. Watson, R. T., Rodhe, H., Oeschger, H. & Siegenthaler, U. in *Climate Change, The IPCC Scientific Assessment* (eds Houghton, J. T., Jenkins, G. J. & Ephraums, J. J.) 1–40 (Cambridge Univ. Press, Cambridge, 1990).

2. Keeling, C. D., Piper, S. C. & Heimann, M. in *Aspects of Climate Variability in the Pacific and Western Americas* (ed. Peterson, D. H.) 305–363 (Geophys. Monogr. 55, American Geophysical Union, Washington DC, 1989).
3. Tans, P. P., Fung, I. Y. & Takahashi, T. *Science* **247**, 1431–1438 (1990).
4. Clais, P. et al. *J. geophys. Res.* **100**, 5051–5070 (1995).
5. Clais, P., Tans, P. P., Trolier, M., White, J. W. C. & Francey, R. J. *Science* **269**, 1098–1102 (1995).
6. Keeling, C. D. et al. in *Aspects of Climate Variability in the Pacific and Western Americas* (ed. Peterson, D. H.) 165–236 (Geophys. Monogr. 55, American Geophysical Union, Washington DC, 1989).
7. Siegenthaler, U. & Sarmiento, J. L. *Nature* **365**, 119–125 (1993).
8. Quay, P. D., Tilbrook, B. & Wong, C. S. *Science* **256**, 74–79 (1992).
9. Keeling, R. F. & Shertz, S. R. *Nature* **358**, 723–727 (1992).
10. Bender, M., Ellis, T., Tans, P. P. & Francey, R. J. and Lowe, D. *Global biogeochem. Cycles* **10**, 9–21 (1996).
11. Conway, T. J. et al. *J. geophys. Res.* **99**, 22831–22855 (1994).
12. Keeling, C. D., Whorf, T. P., Wahlen, M. & van der Plicht, J. *Nature* **375**, 666–670 (1995).
13. Broecker, W. S. & Peng, T.-H. *Nature* **356**, 587–589 (1992).
14. Keeling, R. F., Najjar, R. G., Bender, M. L. & Tans, P. P. *Global biogeochem. Cycles* **7**, 37–67 (1993).
15. Keeling, R. F. & Peng, T.-H. *Phil. Trans. R. Soc. Lond. B* **348**, 133–142 (1995).
16. Heimann, M. *The Global Atmospheric Tracer Model TM2* (Tech. Rep. No. 10, Deutsches Klimarechenzentrum, Hamburg, 1995).
17. Brewer, P. G., Goyet, C. & Dyrssen, D. *Science* **246**, 477–479 (1989).
18. Tarantola, A. and Valette, B. *Rev. Geophys. Space. Phys.* **20**, 219–232 (1982).
19. Denning, A. S., Fung, I. Y. & Randall, D. A. *Nature* **243**, 240–243 (1995).
20. Heimann, M. and Keeling, C. D. in *Aspects of Climate Variability in the Pacific and Western Americas* (ed. Peterson, D. H.) 237–275 (Geophys. Monogr. 55, American Geophysical Union, Washington DC, 1989).
21. Grace, J. et al. *Science* **270**, 778–780 (1995).
22. Six, K. & Maier-Reimer, E. *Global biogeochem. Cycles* (submitted).
23. Keeling, R. F. & Severinghaus, J. P. in *The Carbon Cycle* (eds Wigley, T. M. & Schimel, D.) (Cambridge Univ. Press, in the press).
24. Francey, R. J. et al. *Nature* **373**, 326–330 (1995).
25. Schimel, D. et al. in *Climate Change 1994, Radiative Forcing of Climate Change and an Evaluation of the IPCC IS92 Emission Scenarios* (eds Houghton, J. T. et al.) 35–71 (Cambridge Univ. Press, 1995).
26. Marland, G., Rotty, R. M. & Treat, N. L. *Tellus* **37B**, 243–258 (1985).
27. Andres, R. J., Marland, G., Boden, T. & Bischoff, S. *The Carbon Cycle* (eds Wigley, T. M. & Schimel, D.) (Cambridge Univ. Press, in the press).
28. Hein, R., Crutzen, P. J. & Heimann, M. *Global biogeochem. Cycles* (in the press).
29. Sarmiento, J. L., Orr, J. C. & Siegenthaler, U. *J. geophys. Res.* **97**, 3621–3645 (1992).
30. Watson, A. J., Nightingale, P. D. & Cooper, D. J. *Phil. Trans. R. Soc. Lond. B* **348**, 125–132 (1995).
31. Sarmiento, J. L., Murnane, R. & Le Quéré, C. *Phil. Trans. R. Soc. Lond. B* **348**, 211–219 (1995).
32. Keeling, R. F. thesis, Harvard Univ. (1988).
33. Severinghaus, J. P. thesis, Columbia Univ. (1995).
34. Keeling, R. F. *J. atmos. Chem.* **7**, 153–176 (1988).

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Two mantle-plume components in Hawaiian picrites inferred from correlated Os–Pb isotopes

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OCEAN island basalts (OIBs) are thought to result from the melting of plumes rising from a boundary layer in the mantle, such as the core–mantle boundary or that between the upper and lower mantle at 660 km depth^{1,2}. OIBs display considerable compositional heterogeneity, generally attributed to mixing between components such as depleted mantle, subducted oceanic crust, continental lithospheric mantle and primitive mantle^{3–8}. Although the existence of multiple endmembers in the OIB array is now well established, the relationships between crustal recycling, plume volcanism and deep mantle structure and composition are controversial. We present isotope data for picritic lavas from seven volcanic centres, sampling 2 million years of Hawaiian plume activity⁹, and show that the osmium and lead isotopic compositions form remarkably linear, negative arrays. These

correlations are indicative of binary mixing, but are difficult to explain by combining recycled oceanic crust with depleted mantle. We argue that mixing between two distinct mantle components within the Hawaiian plume provides a better explanation of the isotopic variations of the picrites.

Hawaiian lavas are derived from the largest¹⁰, hottest¹¹ and longest-lived mantle plume currently active on Earth and therefore provide the best opportunity to investigate the nature of mantle plumes and their source regions. The picritic basalts studied here (Table 1) were selected to span the known range of Pb, Sr and Nd isotopic compositions for Hawaiian tholeiites. Our objective is to document the isotopic characteristics of the Hawaiian plume, and to relate these characteristics to the long-term evolution of mantle sources sampled by a single plume. This approach contrasts with previous Os isotopic studies based on reconnaissance sampling of multiple OIB localities^{3,4} or detailed investigations of individual volcanic centres¹².

Hawaiian lavas are isotopically heterogeneous over the length of the chain, but individual volcanic centres tend to have a more restricted range of isotopic ratios^{11,13–15}. The Hawaiian compositional spectrum forms one leg of the OIB isotopic array, and extends from compositions similar to Pacific mid-ocean-ridge basalt (MORB) in lavas from Kilauea and Loihi, towards an endmember component termed Enriched Mantle 1 (EM1)⁵ that is best expressed in lavas from Koolau, Kahoolawe and Lanai. Two classes of model have been proposed to explain the EM1 composition. The Pb, Sr and Nd isotopic compositions of EM1-type OIB are very similar to those predicted for the bulk silicate Earth (crust plus mantle), and suggest a primitive, undifferentiated mantle source^{6,7,11,16}; however trace-element characteristics of Hawaiian lavas argue against direct involvement of primitive mantle in the Hawaiian plume^{17,18}. Alternatively, EM1 represents a three-component mixture of depleted mantle with ancient

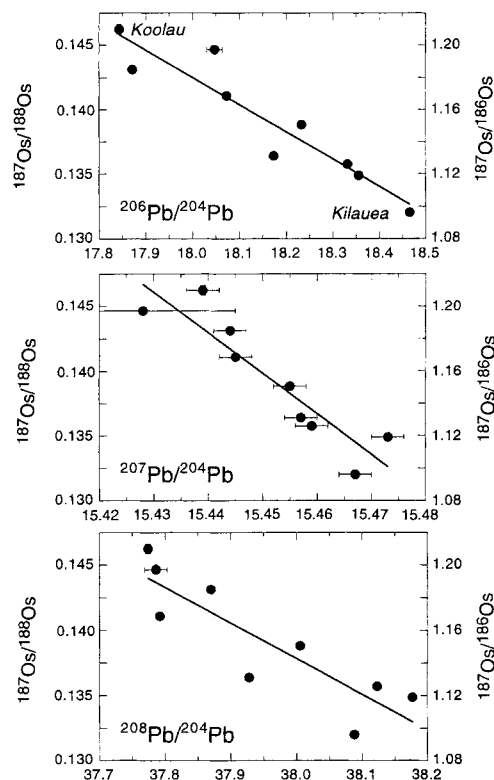


FIG. 1 Pb–Os isotope variation diagrams for Hawaiian picrites. The consistent Os–Pb isotopic correlations for primitive magmas from seven major volcanic centres spanning ~ 2 Myr reflect long-term plume source characteristics. The Koolau and Kilauea endmembers are labelled. Errors are as indicated or are smaller than the symbols.

TABLE 1 Isotopic composition of Hawaiian picritic basalts

Volcano	Sample No.	$^{187}\text{Os}/^{188}\text{Os}$	$^{187}\text{Os}/^{186}\text{Os}$	Os (ng g^{-1})	Re (ng g^{-1})	MgO (wt%)	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{207}\text{Pb}/^{204}\text{Pb}$	$^{208}\text{Pb}/^{204}\text{Pb}$	$^{87}\text{Sr}/^{86}\text{Sr}$	ϵ_{Nd}
Koolau	KOO-CF	$0.1456 \pm .0004$	1.210	0.227	0.181	15.5	17.843	15.439	37.774	0.70414	1.5
Koolau	KOO-17A*	$0.1426 \pm .0002$	1.185	0.465	0.224	21.0	17.871	15.444	37.870	0.70438	0.2
Kahoolawe	KW-25*	$0.1439 \pm .0002$	1.196	0.485	0.271	15.3	18.047	15.428	37.786	0.70416	4.0
Kohala	KOH-1-28	$0.1406 \pm .0001$	1.168	0.792	0.229	20.5	18.072	15.445	37.792	0.70376	6.0
Hualalai	H-5	$0.1384 \pm .0001$	1.150	0.595	0.322	15.0	18.233	15.455	38.005	0.70381	4.7
Mauna Loa	ML-2-50	$0.1361 \pm .0001$	1.131	0.792	0.433	19.9	18.173	15.457	37.928	0.70369	5.5
Loihi	LO-02-04	$0.1347 \pm .0001$	1.119	1.083	0.481	29.9	18.355	15.477	38.177	0.70378	3.9
Loihi	LO-02-02	$0.1355 \pm .0001$	1.126	0.863	0.610	24.8	18.331	15.459	38.123	0.70365	5.5
Kilauea	KIL-1-18	$0.1315 \pm .0001$	1.093	0.296	0.782	13.9	18.465	15.467	38.088	0.70356	7.0

Os isotope ratios have been corrected for Re decay. The corrections are small and within analytical uncertainties. The 2σ errors for Os compositions are indicated in the table, other 2σ are as follows: $^{87}\text{Sr}/^{86}\text{Sr} < \pm 0.00002$, $\epsilon_{\text{Nd}} < \pm 0.2$, $^{206}\text{Pb}/^{204}\text{Pb} \leq \pm 0.003$, $^{207}\text{Pb}/^{204}\text{Pb} \leq \pm 0.003$, $^{208}\text{Pb}/^{204}\text{Pb} \leq \pm 0.01$. Os and Re concentrations were determined by isotope dilution using a mixed ^{185}Re – ^{190}Os spike. Errors in Re and Os concentrations, including uncertainties associated with spike calibration, are $\leq 1\%$. A modified Carius-tube technique was used for sample dissolution and spike equilibration³⁴. Os was extracted by double distillation and Re was isolated using anion-exchange chemistry. Os isotopes were simultaneously measured as OsO_3^- ions in multiple Faraday cups using charge-counting negative thermal ionization mass spectrometry on an ANU-61 mass spectrometer (CC-NTIMS³⁵). Re concentrations were determined by isotope dilution inductively coupled plasma mass spectrometry. A double spike technique was used for fractionation correction of Pb isotope ratios³⁶. Pb, Nd and Sr were analysed on a Finnigan 261 mass spectrometer. Average procedural blanks were: Os, 14 pg; Re, 8 pg; Nd, 35 pg; Sr, 350 pg; Pb, 80 pg. $\epsilon_{\text{Nd}} = [(^{143}\text{Nd}/^{144}\text{Nd})_{\text{sample}}/0.512638 - 1] \times 10^4$.

* MgO, Pb, Nd and Sr data for sample KW-25 are from ref. 37. Sample KOO-17A was discussed in ref. 9; the data presented here are new analyses.

(~2 Gyr old) subducted oceanic crust (MORB plus pelagic sediments) that fortuitously resembles the bulk Earth estimates^{3,19,20}. Ancient subducted sediments with a low U/Pb ratio are necessary to generate the low $^{207}\text{Pb}/^{204}\text{Pb}$ ratios characteristic of EM1.

The picritic basalts analysed here are relatively primitive lavas which contain abundant modal olivine and have high concentrations of MgO and Os (Table 1). They include both subaerial lavas and dredge samples collected from offshore rift zones surrounding the islands. Previous studies of more evolved basalts^{12,21} have found an inverse correlation between elevated $^{187}\text{Os}/^{188}\text{Os}$ ratios and Os concentrations for Os-poor samples ($\leq 0.05 \text{ ng g}^{-1}$). Such trends indicate alteration of the isotopic compositions by magmatic processes, including assimilation of oceanic crust and sediment during magma ascent and hydrothermal interaction with sea water. In contrast, the high Os contents of picrites (0.22–1.08 ng g^{-1} , Table 1) render their Os isotopic compositions resistant to change by high-level magmatic processes and thus representative of their mantle sources.

The new Os isotopic data (Table 1) are in agreement with previously determined compositions for Kilauea²², Loihi³ and Mauna Loa²³; however, we have significantly extended the range of Os isotopic compositions for high-Mg Hawaiian basalts to more radiogenic values by including other volcanic centres, in particular Kahoolawe and Koolau. Osmium isotopic compositions for the Hawaiian chain span a wide range, from $^{187}\text{Os}/^{188}\text{Os}$ of 0.1312 ($^{187}\text{Os}/^{186}\text{Os} = 1.09$) in Kilauea picrites to 0.1456 ($^{187}\text{Os}/^{186}\text{Os} = 1.21$) for those from Koolau. The data show strong correlations between the Os, Pb, Sr and Nd isotopic compositions (Fig. 1; Table 1). In three dimensions (Fig. 2) the Os–Pb–Sr data form a line, not a plane, indicating that the array is generated by mixing between two components. This observation places strong constraints on the nature of any recycled component in the Hawaiian plume, requiring the crustal endmember to have fixed proportions of basalt:sediment for at least the last 2 million years.

Multi-isotope mixing diagrams are often used to evaluate potential OIB sources, but in this case the enhanced utility of the Re–Os isotopic system lies in the distinctive geochemical behaviour of Os. During melting of mantle peridotite, Pb, and to a slightly lesser extent Sr and Nd, behave incompatibly and are preferentially concentrated in the melt relative to the residual solid. In contrast, Os is largely retained in residual phases. Because of this divergent geochemical behaviour, crustal materials and their melts will have concentration ratios of Sr, Nd and Pb to Os that are significantly greater than those of the depleted

mantle, with Pb/Os exhibiting the most extreme differences between mantle and crustal components. Binary mixing arrays have the general form of hyperbolae²⁴, and for any two fixed endpoints the degree of curvature q is governed by the ratio of the concentrations of chemical species in each endmember. For Pb–Os isotopic mixtures, $q = [(\text{Pb}/\text{Os})_{\text{component1}}/(\text{Pb}/\text{Os})_{\text{component2}}]$. It is only when ratios are subequal, ($q \approx 1$), that the hyperbolic trend reduces to a straight line; as q deviates from 1, either positively or negatively, the degree of curvature increases.

In Fig. 3 we compare the Hawaiian picrite Os and Pb isotopic compositions with hyperbolae calculated for mixtures of various components. Mixtures of ~70% of MORB-source depleted mantle (DM), which is similar, but not identical, to the Kilauea endmember, and ~30% of a recycled component, which itself is composed of ~2-Gyr-old MORB (97%) plus pelagic sediment (3%), can reproduce the isotopic compositions of Koolau picrites. The strongly curved hyperbola ($q = 0.0006$) of such a mixture, however, fails to match the linear Os–Pb data array (Fig. 3), reflecting the much greater Pb/Os ratio of the recycled component relative to DM. Although the compositional range of recycled sediments and MORB components is not precisely known, this conclusion is robust for all plausible isotopic compositions and concentrations. In general, recycling of basalts generates positive Pb–Os isotopic correlations in mixing diagrams (see, for example, ref. 8). It is only for the special case involving ancient, low-U/Pb

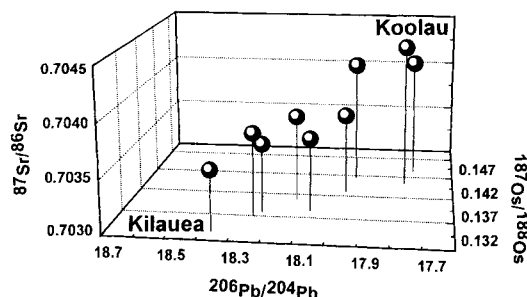


FIG. 2 Pb, Sr and Os isotopic correlation for Hawaiian picrites. The data define a line in three dimensions, indicating that the isotopic variation is caused by mixtures of only two components. The Kilauea picrites are from a depleted-mantle source, isotopically similar to Pacific MORB, whereas the Koolau picrites have Pb and Sr isotopic compositions similar to estimated bulk silicate Earth values, but with more radiogenic Os isotopic compositions.

sediments plus associated MORB that negative correlations can be produced and these will be strongly curved. A two-step process involving the formation of an enriched intermediate mantle component by bulk addition of recycled oceanic crust to DM, and then mixing this intermediate component with additional DM, results in similarly curved hyperbolae. We conclude that the Os–Pb isotopic array found in Hawaiian picrites cannot be readily reproduced by binary mixtures of depleted mantle and recycled oceanic crust, regardless of its age or sediment component.

Alternatively, the isotopic compositions of Hawaiian picrites can be accounted for by mixing between two mantle components with broadly similar bulk compositions, but which are isotopically distinct. Curve B (Fig. 3) shows the mixing array for depleted mantle with a modified primitive mantle. The difference in Pb/Os concentration ratios between depleted mantle and undepleted mantle results in a slightly curved array, but approximates the picrite data trend. However, the picrite Pb–Os array is best reproduced if $q \approx 1$, requiring subequal Pb/Os concentration ratios in the two plume components (Curve C, Fig. 3). From this we infer that both endmembers must have very similar ratios of incompatible to compatible elements, but with one having a radiogenic Os isotopic composition. Furthermore, the high-MgO glasses from Kilauea²⁵, and the abundance of olivine phenocrysts with atomic $\text{Mg}/(\text{Mg} + \text{Fe}) = 0.88\text{--}0.90$ in picrites from Mauna Loa, Hualalai and Koolau (ref. 10 and M.D.N., unpublished data) indicate that both of the parental components are fundamentally peridotitic in bulk composition.

The provenance of plume sources is only poorly constrained. Tomography²⁶ and geodynamic models² suggest that plumes

originate in the deep mantle, at or below the 660-km seismic discontinuity. The isotopic similarity between the Kilauea picrites and Pacific MORB raises the possibility of mixing of upper-mantle material with the rising plume; however, fluid-dynamic models suggest that little of the upper mantle will be entrained in a plume tail^{1,27} such as is believed to underlie Hawaii. Oxygen isotopic compositions of Hawaiian basalts are also distinct from those of MORB²⁸. Therefore, the physical, chemical and isotopic constraints for picrites from both Koolau and Kilauea argue for two deep-mantle components in the plume. The Sr–Nd–Pb isotopic compositions of the Koolau endmember require a time-integrated history with parent–daughter ratios close to those of the bulk silicate Earth, as would be expected of a primitive component equivalent to the undepleted lower mantle^{7,11}; accordingly, primitive mantle contributions have been proposed specifically for Hawaii^{6,7,9,29}. Although certain important trace-element ratios such as Nb/Th do not support the direct contribution of primitive mantle to Hawaiian basalts^{17,18}, at least some trace-element ratios can be fractionated by relatively recent events and do not necessarily reflect the long-term characteristics of their source. The Os isotopic compositions of Koolau picrites require a source with an average Re/Os ratio at least 65% higher than in upper mantle that evolved with an approximately chondritic Re/Os since at least 3.8 Gyr ago³⁰. Although it has been generally assumed that the lower mantle has chondritic Re–Os characteristics, it is not necessarily constrained to have the same Os evolution as the upper mantle. A lower-mantle process that could significantly fractionate Re from Os while maintaining high Os concentrations and without affecting incompatible element ratios such as Sm/Nd, U/Pb, Th/Pb and Rb/Sr remains speculative, but possible mechanisms may include interaction between the core and mantle³¹ or heterogeneous accretion³².

A variant of the recycling model involving refertilization of the depleted mantle with partial melts from subducted slabs, possibly at the transition zone³³ (400–660 km depth), could, in principle, produce the Koolau compositions, although the observed isotopic compositions of Hawaiian lavas would require very precise compositional constraints on such a process. Trace-element fractionations during hydrothermal alteration of the oceanic crust, subduction-zone processing and deep-mantle melting are currently insufficiently constrained to evaluate quantitatively such a model. □

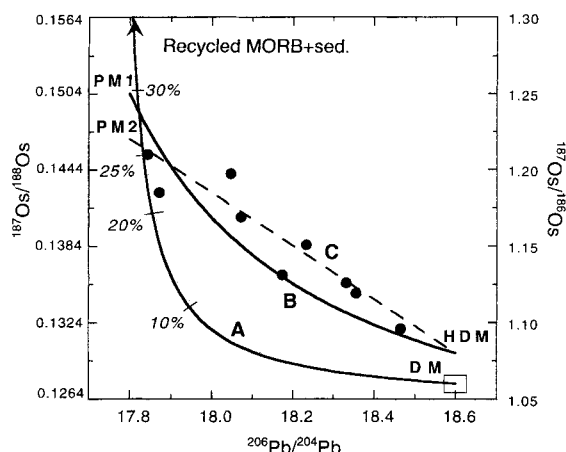


FIG. 3 Pb–Os mixing curves for various potential endmembers. Mixtures of recycled material with depleted mantle (DM) produce strongly curved hyperbolae (curve A). The following parameters apply^{3,19}: ancient recycled component (2-Gyr MORB (97%) + pelagic sediment (3%)) with $\text{Pb} = 1,275 \text{ p.p.b.}$, $^{206}\text{Pb}/^{204}\text{Pb} = 17.76$, $\text{Os} = 0.06 \text{ p.p.b.}$, $^{187}\text{Os}/^{188}\text{Os} = 3.25$ ($^{187}\text{Os}/^{186}\text{Os} = 27$); DM, $\text{Pb} = 40 \text{ p.p.b.}$, $^{206}\text{Pb}/^{204}\text{Pb} = 18.6$, $\text{Os} = 3.4 \text{ p.p.b.}$, $^{187}\text{Os}/^{188}\text{Os} = 0.1275$ ($^{187}\text{Os}/^{186}\text{Os} = 1.06$). The labelled percentages indicate the amount of recycled component in the mixture. Curve B, the Hawaii picrite data are more closely approximated by mixtures of a modified depleted mantle component (HDM) with undepleted (primitive) mantle (PM1), if the undepleted mantle has a much more radiogenic Os isotopic composition than the upper mantle. Mixing parameters for PM1: $^{206}\text{Pb}/^{204}\text{Pb} = 17.8$, $^{187}\text{Os}/^{188}\text{Os} = 0.1504$ ($^{187}\text{Os}/^{186}\text{Os} = 1.25$), $\text{Os} = 3.4 \text{ p.p.b.}$, $\text{Pb}/\text{Os} = 41.18$; for HDM: $^{206}\text{Pb}/^{204}\text{Pb} = 18.6$, $^{187}\text{Os}/^{188}\text{Os} = 0.1300$ ($^{187}\text{Os}/^{186}\text{Os} = 1.08$), $\text{Os} = 3.4 \text{ p.p.b.}$, $\text{Pb}/\text{Os} = 11.76$. The best fit to the data, curve C, requires mixing in the plume source of two endmembers with equal Pb/Os concentration ratios. This requires two mantle components, the Koolau component possibly generated by refertilization of depleted mantle or modified primitive material. Curve C optimized parameters: HDM as for curve B; modified PM2 as for curve B but with $\text{Pb}/\text{Os} = 11.76$ and $^{187}\text{Os}/^{188}\text{Os} = 0.1468$ ($^{187}\text{Os}/^{186}\text{Os} = 1.22$).

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- Griffiths, R. W. & Campbell, I. H. *Earth planet. Sci. Lett.* **99**, 66–78 (1991).
- Duncan, R. A. & Richards, M. A. *Rev. Geophys.* **29**, 31–50 (1991).
- Roy-Barman, M. & Allège, C. J. *Earth planet. Sci. Lett.* **129**, 145–161 (1995).
- Hauri, E. H. & Hart, S. R. *Earth planet. Sci. Lett.* **114**, 353–371 (1993).
- Zindler, A. & Hart, S. R. *Rev. Earth planet. Sci.* **14**, 493–571 (1986).
- White, W. M. *Geology* **13**, 115–118 (1985).
- Hart, S. R. *Earth planet. Sci. Lett.* **90**, 273–296 (1988).
- Marcantonio, F., Zindler, A., Eliot, T. & Staudigel, H. *Earth planet. Sci. Lett.* **133**, 397–410 (1995).
- Roden, M. F., Trull, T., Hart, S. R. & Frey, F. A. *Geochim. cosmochim. Acta* **58**, 1431–1440 (1994).
- Sleep, N. H. *J. geophys. Res.* **95**, 6715–6736 (1990).
- Garcia, M. O., Hulsebosch, T. P. & Rhodes, J. M. *Mauna Log Revealed* 219–240 (Geophys. Monogr. 92, Am. Geophys. Union, Washington DC, 1995).
- Martin, C. E., Carlson, R. W., Shirey, S. B., Frey, F. A. & Chen, C.-Y. *Earth planet. Sci. Lett.* **128**, 287–301 (1994).
- West, H. B., Gerlach, D. C., Leeman, W. P. & Garcia, M. O. *Nature* **330**, 216–219 (1987).
- Stille, P., Unruh, D. M. & Tatsumoto, M. *Geochim. cosmochim. Acta* **50**, 2303–2319 (1986).
- Kurz, M. D. *Phil. Trans. R. Soc. Lond. A* **342**, 91–103 (1993).
- Schilling, J. G. *Nature* **242**, 565–571 (1973).
- Frey, F. A., Garcia, M. O. & Roden, M. F. *Geochim. cosmochim. Acta* **58**, 1441–1462 (1994).
- Hofmann, A. W. *Chem. Geol.* **57**, 17–30 (1986).
- Weaver, B. L. *Earth planet. Sci. Lett.* **104**, 381–397 (1991).
- Chauvel, C., Hofmann, A. W. & Vidal, P. *Earth planet. Sci. Lett.* **110**, 99–119 (1992).
- Reisberg, L. et al. *Earth planet. Sci. Lett.* **120**, 149–167 (1994).
- Martin, C. E. *Geochim. cosmochim. Acta* **55**, 1421–1434 (1991).
- Hauri, E. H., Lassiter, J. C., DePaolo, D. J. & Rhodes, J. M. *J. geophys. Res.* (in the press).
- Albarède, F. *Introduction to Geochemical Modeling* (Cambridge Univ. Press, 1995).
- Clague, D. A., Weber, W. S. & Dixon, J. E. *Nature* **353**, 553–556 (1991).
- Romanowicz, B. *Earth planet. Sci. Lett.* **128**, 113–121 (1994).
- Hart, S. R., Hauri, E. H., Oschmann, L. A. & Whitehead, J. A. *Science* **256**, 517–520 (1992).
- Garcia, M. O., Muenow, D. W., Aggrey, K. E. & O'Neill, J. R. *J. geophys. Res.* **94**, 10525–10538 (1989).
- Honda, M., McDougall, I., Patterson, D. B., Douglis, A. & Clague, D. A. *Geochim. cosmochim. Acta* **57**, 859–874 (1993).
- Bennett, V. C. & Esat, T. M. *Eos (abstr.)* **76**, 45 (1995).

31. Walker, R. J. & Morgan, J. W. *Science* **269**, 819–821 (1995).
32. Newsom, H. E. in *Origin of the Earth* (eds Newsom, H. E. & Jones, J. H.) 273–288 (Oxford Univ. Press, New York, 1990).
33. Ringwood, A. E. *Geochim. cosmochim. Acta* **55**, 2083–2110 (1991).
34. Shirey, S. B. & Walker, R. J. *Analyt. Chem.* **34**, 2136–2141 (1995).
35. Esat, T. M. *Int. J. Mass Spectrom. Ion Proc.* **148**, 159–170 (1995).
36. Woodhead, J., Volker, F. & McCulloch, M. T. *Analyst* **120**, 35–39 (1995).
37. Leeman, W. P., Gerlach, D. C., Garcia, M. O. & West, H. B. *Contr. Miner. Petrol.* **116**, 62–77 (1994).

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A late Neanderthal associated with Upper Palaeolithic artefacts

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THE French site of Arcy-sur-Cure is a key locality in documenting the Middle–Upper Palaeolithic transition in Europe. Reliable attribution of the fragmentary hominid fossils associated with its early Upper Palaeolithic Châtelperronian industry has not been possible. Here we report the first conclusive identification of one of these fossils as Neanderthal on the basis of newly discovered derived features of the bony labyrinth. Dated at about thirty-four thousand years (34 kyr) ago, the fossil is representative of the youngest known Neanderthal populations, and its archaeological context indicates that these hominids used a rich bone industry as well as personal ornaments. The evidence supports the hypothesis of a long term coexistence with technocultural interactions between the first modern humans and the last Neanderthals in Europe. However, the complete absence of the derived Neanderthal traits in labyrinths of modern Upper Palaeolithic specimens from western Europe argues against phylogenetic continuity between the two populations in this region.

The Châtelperronian, an early Upper Palaeolithic industry known from northern Spain and central and southwestern France, is of the utmost importance for our understanding of the Middle–Upper Palaeolithic transition in western Europe. In contrast to the contemporary Aurignacian, the Châtelperronian appears to have emerged from the local Mousterian industries, associating Middle Palaeolithic artefacts with Upper Palaeolithic elements such as blade technology and a developed bone industry^{1–4}. The Châtelperronian and Aurignacian may have emerged at multiple locations as independent parallel technological inventions⁵, perhaps in association with the predominantly local evolution of modern humans, with significant genetic input from Neanderthals^{6,7}. Alternatively, modern humans may have invaded Neanderthal territory, introducing the Aurignacian. According to this view, the Châtelperronian could have resulted from an acculturation process of the last western Neanderthals during a variable period of coexistence with modern humans^{1,2,8}.

The identity of the Châtelperronian tool-maker is crucial to the assessment of the two scenarios. Thus far, the only firm fossil evidence comes from Saint-Césaire (Charente-Maritime,

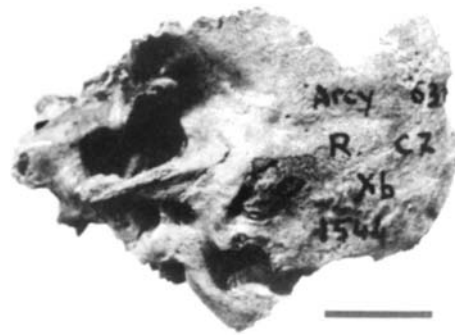


FIG. 1 Lateral view of the Arcy-sur-Cure juvenile temporal bone. Scale bar, 10 mm.

France), where a partial Neanderthal skeleton is dated at 36 ± 3 kyr before present (BP)^{9,10}. The only other Châtelperronian site with well associated hominid remains and the northernmost appearance of this industry, is Arcy-sur-Cure (Grotte du Renne) in the River Yonne Basin, 35 km south-east of Auxerre (France). Establishing the affiliation of these fossils is of particular interest because the Arcy assemblage is one of the latest known occurrences of the Châtelperronian, persisting beyond 33 kyr BP (refs 11,12). Moreover, it is unique in that it includes a rich bone and ivory industry and personal ornaments (pierced or grooved animal teeth and ivory rings), classically referred to the Upper Palaeolithic^{13,14}. Although the morphology of some hominid teeth suggested a possible non-modern assignment¹⁵, the fragmentary nature of the fossils have precluded conclusive identification², including doubt over the hominid status of some of these specimens. The Châtelperronian layer Xb at Arcy yielded a previously undescribed hominid temporal bone fragment, which preserves parts of the petrous, tympanic and mastoid portions (Fig. 1). Judging by its development, it belonged to an individual of about one year old, which makes taxonomic assignment on the basis of the usual criteria very difficult. Layer Xb is dated at 33.820 ± 0.720 yr BP by ¹⁴C (ref. 16), which is consistent with the ¹⁴C ages of underlying and overlying deposits¹⁷.

The morphology of the bony labyrinth within the temporal bone has the potential to provide information about hominid phylogenetic relationships¹⁸. We explored the comparative morphology of this structure in Neanderthals and modern humans in an attempt to establish the affinities of the Arcy specimen. Methods for visualization of the bony labyrinth in extant and fossil hominid crania using high-resolution computed tomography have been reported^{19,20}. The specimens that were studied are described in the legend to Fig. 3. The labyrinth gains its adult morphology long before birth, and direct comparison can therefore be made between adult and non-adult specimens²¹.

The radii of curvature of the semicircular canals indicate that

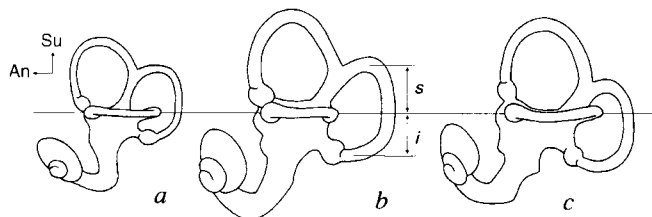


FIG. 2 Lateral aspect of the left labyrinths of a, *Pan paniscus*; b, modern *Homo sapiens*; and c, the Neanderthal cranium La Ferrassie 1. An, anterior; Su, superior. The sagittal labyrinthine index indicates what percentage of the posterior canal is situated inferiorly to the plane of the lateral canal ($i/s + i \times 100$). Exact definitions and method of taking the measurements are given in refs 18, 20 and 21.

Neanderthals have smaller anterior and posterior canals than modern humans, both extant and a sample of French Upper Palaeolithic specimens (Table 1a). The smaller canals in Neanderthals are particularly striking if it is considered that body mass, which is positively correlated with canal size^{18,21}, was probably higher in Neanderthals than in modern humans²². The labyrinth of Neanderthals is further characterized by a markedly inferiorly-positioned posterior semicircular canal relative to the plane of the lateral canal (Fig. 2 and Table 1a). In great apes and hominids, the position and size of the posterior canal are highly correlated: the larger the canal the more inferior is its position (Fig. 3). Neanderthals in contrast do not follow this trend. Rather they show an inferiorly-positioned but relatively small posterior canal.

Homo erectus s.l. is not significantly different from modern humans in any of the traits characterizing the Neanderthal morphology (Table 1a and Fig. 3). Assuming that *H. erectus s.l.* is ancestral to all later forms of the genus *Homo* therefore implies that the Neanderthal labyrinthine morphology should be interpreted as derived relative to *H. erectus s.l.* and modern humans.

A trend of anterior and posterior semicircular canal enlargement, identified throughout hominid evolution, has been functionally linked with the emergence of obligatory bipedalism¹⁸. That Neanderthals as habitual bipeds nevertheless have relatively small canals demonstrates that the link between canal size, head movements and locomotor behaviour is complex, possibly involving factors such as neck and ocular motility. Differential brain development has been identified as a likely factor underlying remodelling of the labyrinthine shape^{19,21}. In this light, a possible link between the inferiorly positioned posterior canal and the development of the typically large and platycephalic brain in Neanderthals may warrant further investigation. Assessing the labyrinth in European Middle Pleistocene hominids will contribute to our understanding of the phylogeny of the derived Neanderthal characters.

The Arcy temporal specimen can be identified as Neanderthal on the basis of the full suite of Neanderthal features shown by its labyrinth (Table 1a and Figs 2, 3). A multivariate analysis of the data fully supports this diagnosis (Table 1b, c). Dated at about 34 kyr BP, the Arcy hominid joins the specimens from Zafarraya (southern Spain)²³ as the youngest known Neanderthal fossils,

TABLE 1 Comparison of labyrinthine measurements

(a)	ASC-R		PSC-R		LSC-R		SLI	
	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
<i>Homo erectus</i>	3.2	0.06	3.1	0.25	2.1*	0.23	51**	9.1
Upper Palaeolithic	3.3*	0.13	3.1	0.23	2.5	0.12	42***	8.9
<i>Homo sapiens</i>	3.2**	0.24	3.1*	0.30	2.3***	0.21	51***	7.1
Neanderthals	3.0	0.22	2.9	0.19	2.5	0.20	68	4.9
Arcy-sur-Cure	2.9		2.8		2.5		64	

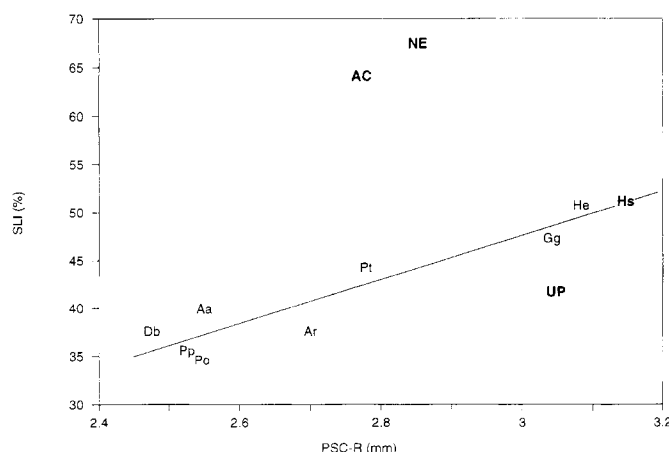
(b)		(c)	
UP	7.9	AC-NE < AC-Hs	
AC	24.7	AC-NE < AC-UP	
NE	14.8	AC-NE < AC-He	
He	9.4	NE-AC < NE-Hs	
Hs	UP	NE-AC < NE-UP	
	AC	NE-AC < NE-He	
	NE	UP-NE > UP-Hs	
		UP-NE < NE-Hs	

a, The radii of curvature of the anterior, posterior and lateral semicircular canals (ASC-R, PSC-R, LSC-R, respectively), defined in refs 18 and 20, given in millimetres, and the sagittal labyrinthine index (SLI), defined in Fig. 2, given in percentages. The mean and standard deviation are given. Statistical significance of the difference from the mean of the Neanderthal sample (t-test) is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

b, The squared Mahalanobis distances (D^2) between the five groups of a, based on seven principle components of seven variables (the six original measurements from which the canal radii of curvature were calculated^{18,20} plus distance 'i' indicated in Fig. 2). See Fig. 3 for the codes of the groups. Tabun C1 is not included, owing to one missing variable. All D^2 values are significant (Hotelling test, $P < 0.01$), except the one between Arcy (AC) and Neanderthals (NE). c, Significantly different Mahalanobis distances relevant to this study (Steinemann test²⁰, $P < 0.05$). For example, AC-NE < AC-Hs means that the distance between AC and NE is significantly smaller than between AC and Hs. > indicates larger distances. The top six comparisons imply that the Arcy specimen and the Neanderthal sample are the most closely related, and the bottom two that the Upper Palaeolithic sample is not intermediate between extant modern humans and Neanderthals.

giving new evidence of the long-term survival of these hominids. Associating the Aurignacian, known from western Europe to be as early as 40 kyr²⁴⁻²⁶, with modern humans implies their coexistence with the last Neanderthals over a significant period. Nevertheless, neither the specimens from Zafarraya²³, nor the labyrinth of the Arcy Neanderthal show any trend towards a more modern human morphology. The modern Upper Palaeolithic labyrinth, on the other hand, seems even more distinct from the derived Neanderthal pattern than that of extant modern humans (Table 1b, c). In the disagreement between studies suggesting any degree of morphological continuity between Neanderthals and modern humans^{6,7}, and those proposing a reproductive barrier between the two populations^{8,27,28}, the evidence presented here is most compatible with the latter view, at least as far as western Europe is

FIG. 3 The relationship between the radius of curvature of the posterior semicircular canal (PSC-R, in millimetres) and the sagittal labyrinthine index (SLI, in percentages). NE, Neanderthals (La Chapelle aux Saints, La Ferrassie 1 and 2, La Quina 5 and H27, Pech de l'Aze, Gibraltar 1 and 2, Tabun C1); UP, Upper Palaeolithic (Cro Magnon 1, Abri Pataud 1 and 3, Laugerie Basse 1); AC, Arcy-sur-Cure (partial temporal bone); He, *Homo erectus* (OH 9, Sangiran 2, Sangiran 4); Aa, *Australopithecus africanus* (Taung, Sts 5, Sts 19, MLD 37/38); Ar, *Australopithecus robustus* (SK 46, SK 47, SK 879); Db, *Dryopithecus brancoi* (RUD 77); Hs, *Homo sapiens* ($n = 53$); Pt, *Pan troglodytes* ($n = 7$); Pp, *Pan paniscus* ($n = 6$); Gg, *Gorilla gorilla* ($n = 6$); Po, *Pongo pygmaeus* ($n = 7$). The least-square regression line is given for the sample excluding the Neanderthal, Upper Palaeolithic and Arcy-sur-Cure specimens. Correlation coefficient, $r = 0.946$; slope, 23.05; intercept, -21.52.



concerned. The strikingly derived nature of the Neanderthal labyrinthine features, already present before birth, and the apparent lack of continuity with modern human morphology could be seen as an argument in support of distinguishing between Neanderthals and modern humans at the species level.

The evidence from Arcy indicates that advanced Châtelperronian industries were used by late Neanderthals, suggesting a high degree of acculturation. The association of the Arcy Neanderthal with personal ornaments so similar to those found in contemporary and nearby Aurignacian layers^{14,29} questions the nature of the cultural interactions with modern humans. At least in the case of these specific objects, we may be facing evidence of a trading process rather than the result of technical imitation of modern human technology by Neanderthals. □

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- Demars, P. Y. & Hublin, J. J. *ERAUL Liege* **34**, 29–42 (1989).
- Harold, F. B. in *The Human Revolution* (eds Mellars, P. A. & Stringer, C. B.) 677–713 (Edinburgh University Press, Edinburgh, 1989).
- Mellars, P. A. *Phil. Trans. R. Soc. Lond. B* **337**, 225–234 (1992).
- Pelegri, J. *Technologie Lithique: le Châtelperronien de Roc-de-Combe (Lot) et de la Côte (Dordogne)* (Paris, CNRS Cahiers du quaternaire, 1995).
- Straus, L. G. *Evol. Anthropol.* **2**, 195–198 (1993–1994).
- Clark, G. A. & Lindly, J. M. in *The Human Revolution* (eds Mellars, P. A. & Stringer, C. B.) 621–676 (Edinburgh University Press, Edinburgh, 1989).
- Wolpoff, M. & Frayer, D. *Nature* **356**, 200–201 (1992).
- Stringer, C. & Grün, R. *Nature* **351**, 701–702 (1991).
- Léveque, F. & Vandermeersch, B. *C.R. Acad. Sc. Paris* **291**, 187–189 (1980).
- Mercier, N. et al. *Nature* **351**, 735–739 (1991).
- Leroyer, C. & Leroi-Gourhan, A. *Bull. Soc. Prehist. Fr.* **80**, 41–44 (1983).
- Leroyer, C. *ERAUL Liege* **35**, 103–108 (1988).
- Baffier, D. & Julien, M. in *Paléolithique Moyen Récent et Paléolithique Supérieur Ancien en Europe* (ed. Fairizy, C.) 329–334 (Musée de Préhistoire d'Ile de France, Nemours, 1990).

- Taborin, Y. in *Paléolithique Moyen Récent et Paléolithique Supérieur Ancien en Europe* (ed. Fairizy, C.) 335–244 (Musée de Préhistoire d'Ile de France, Nemours, 1990).
- Leroi-Gourhan, A. *Ann. Paléont.* **44**, 87–148 (1958).
- Hedges, R. E. M., Housley, R. A., Bronk Ramsey, C. & Van Klinken, G. J. *Archaeometry* **36**, 337–374 (1994).
- Girard, M., Miskovsky, J. C. & Evin, J. in *Paléolithique Moyen Récent et Paléolithique Supérieur Ancien en Europe* (ed. Fairizy, C.) 295–303 (Musée de Préhistoire d'Ile de France, Nemours, 1990).
- Spoor, F., Wood, B. & Zonneveld, F. *Nature* **369**, 645–648 (1994).
- Spoor, C. F. & Zonneveld, F. W. *Cour. Forsch. Senckenberg* **171**, 251–256 (1994).
- Spoor, C. F. & Zonneveld, F. W. *J. Anat.* **186**, 271–286 (1995).
- Spoor, C. F. *The Comparative Morphology and Phylogeny of the Human Bony Labyrinth* (Utrecht University, Utrecht, 1993).
- Ruff, C. B. *Yb. of Phys. Anthropol.* **37**, 65–107 (1994).
- Hublin, J. J., Barroso Ruiz, C., Medina Lara, P., Fontugne, M. & Reyss, J.-L. *C.R. Acad. Sc. Paris* **321** 11a, 931–937 (1995).
- Bischoff, J. L., Soler, N., Maroto, J. & Julia, R. J. *Archaeol. Sci.* **16**, 553–576 (1989).
- Cabrera-Valdes, V. & Bischoff, J. L. *J. Archaeol. Sci.* **16**, 577–584 (1989).
- Bischoff, J. L. et al. *J. Archaeol. Sci.* **21**, 541–551 (1994).
- Howell, F. C. in *Origins of Anatomically Modern Humans* (eds Nitecki, M. H. & Nitecki, D. V.) 253–319 (Plenum, New York, 1994).
- Rak, Y. in *Species, Species Concepts and Primate Evolution* (eds Kimbel, W. H. & Martin, L. B.) 523–536 (Plenum, New York, 1994).
- Lejeune, M. *L'art Mobilier Paléolithique et Mésolithique de Belgique (Artefacts 4)* (Arc, Viroinval, 1987).
- Van Vark, G. N. in *Multivariate Statistical Methods in Physical Anthropology* (eds Van Vark, G. N. & Howells, W. W.) 323–349 (Riedel, Dordrecht, 1984).

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Continental breakup and the ordinal diversification of birds and mammals

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THE classical hypothesis for the diversification of birds and mammals proposes that most of the orders diverged rapidly in adaptive radiations after the Cretaceous/Tertiary (K/T) extinction event 65 million years ago^{1–3}. Evidence is provided by the near-absence of fossils representing modern orders before the K/T boundary^{4,5}. However, fossil-based estimates of divergence time are known to be conservative because of sampling biases⁶, and some molecular/time estimates point to earlier divergences among orders^{7–10}. In an attempt to resolve this controversy, we have estimated times of divergence among avian and mammalian orders with a comprehensive set of genes that exhibit a constant rate of substitution. Here we report molecular estimates of divergence times that average about 50–90% earlier than those predicted by the classical hypothesis, and show that the timing of these divergences coincides with the Mesozoic fragmentation of emergent land areas. This suggests that continental breakup may have been an important mechanism in the ordinal diversification of birds and mammals.

Molecular time estimation of evolutionary divergence requires genes that are evolving at a relatively constant rate, which often limits the number of sequences available for analysis^{8,9}. Fortunately, the widespread use of model organisms for genetic

FIG. 1 Relationship between the evolutionary histories of mammals and birds and events in earth history during the Mesozoic and Cenozoic. *a*, Fossil record of the orders of eutherian mammals and birds⁴ (solid line, certain; broken line, uncertain); avian taxonomy from ref. 29. *b*, Molecular estimates of divergence times based on constant-rate genes (from Table 1); the number of genes (constant rate/total) is in parentheses; filled circles, mean; horizontal bar, $\pm$ s.e.; thin vertical line, median. *c*, Land breakup (fission) and fusion events determined from reconstructions of emergent land areas during the last 250 Myr (ref. 30); only emergent land areas $> 3 \times 10^6$ km² (about the size of India) are considered here, although the same pattern is evident if smaller areas (say, the area of Madagascar) are considered. *d*, Total emergent land area exposed at different times during the last 250 Myr (ref. 30).

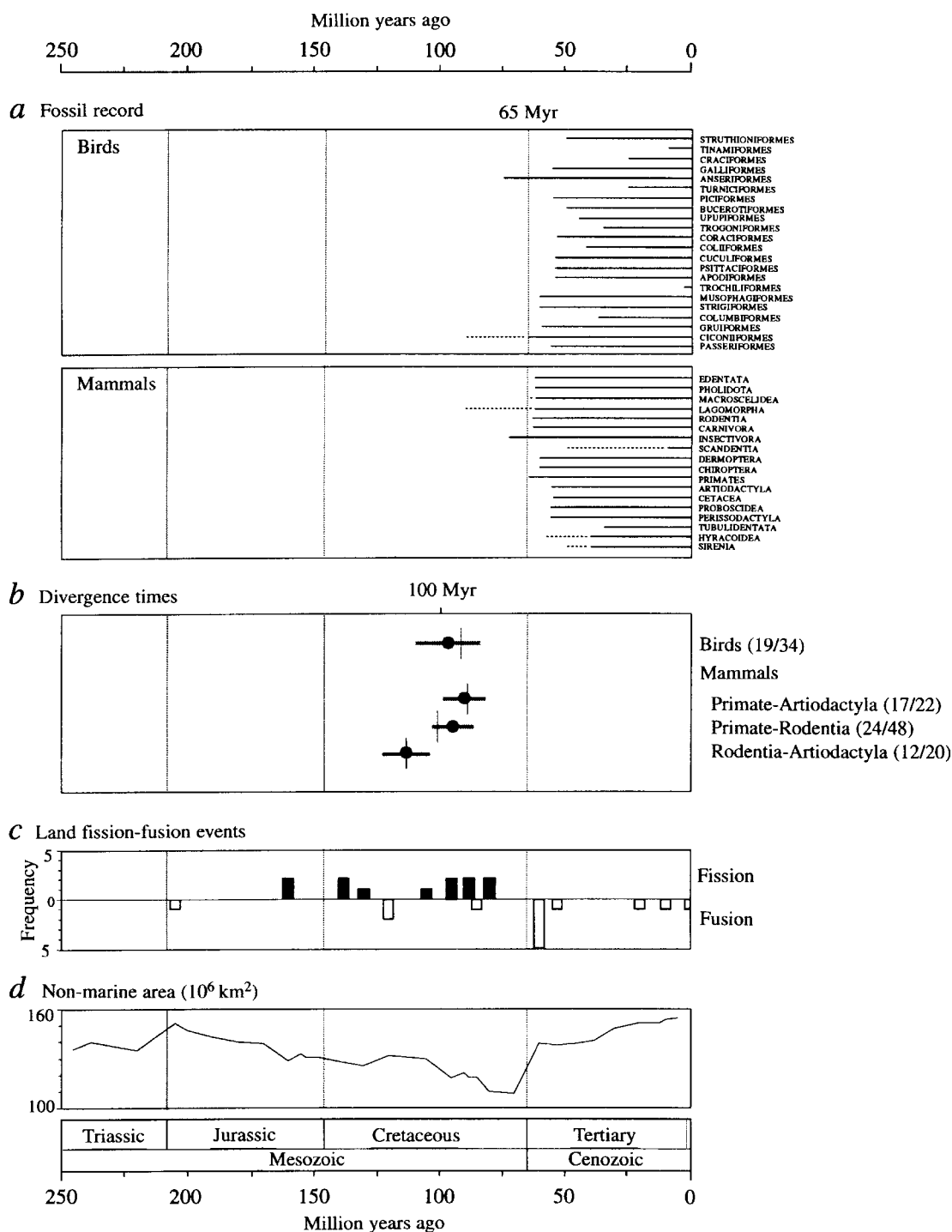
METHODS. For avian comparisons (in *b*), the nuclear genes used and taxa are (g, galliform; a, anseriform; c, columbiform; s, struthioniform): acylphosphatase-muscle_{ga}, alcohol dehydrogenase_{gs}, α -crystallin_{gacs}, α -globin_{gacs}, argininosuccinate lyase_{ac}, β 2 thyroid hormone receptor_{hm}, β -globin_{gacs}, cytochrome c_{gacs}, embryonic α -globin_{ga}, histone 1_{ga}, lactate dehydrogenase B_{ga}, lysozyme c_{ga}, malic enzyme_{ac}, MX protein_{ga}, prolactin receptor_{gc}, and somatotropin_{ga} (for all genes, sequences of a mammal and an outgroup (or paralogous sequence) also were used). For mammalian comparisons, the nuclear genes used and taxa are (h, *H. sapiens*; m, *M. musculus*; b, *B. taurus*): acetylcholine receptor α _{hmb}, acetylcholine receptor δ _{hmb}, acetylcholine receptor γ _{hm}, α -globin_{hmb}, annexin I_{hm}, Bc12-Ig fusion gene_{hm}, B-myb_{hmb}, casein kinase II α _{hb}, CD18-integrin β -2_{mb}, CDC2_{hmb}, c-kit proto-oncogene_{hm}, C-myb_{hmb}, connexin 43_{hmb}, cyclin A_{hmb}, cyclophilin B_{mb}, c-yes protein_{hm}, E-cadherin_{hm}, erythroblast virus oncogene homologue 2_{hm}, fatty-acid-binding protein_{hmb}, focal adhesion kinase_{hm}, follistatin_{hmb}, GATA-3_{hm}, histone H2A_{hm}, lactate dehydrogenase A_{hmb}, lamin B1_{hm}, midline protein_{hm}, MOS proto-oncogene_{hm}, muscle pyruvate kinase_{hm}, myelin proteolipid protein_{hmb}, Myf-5_{hm}, Na⁺/K⁺ ATPase β -2_{hm}, NAD⁺ ADP-ribosyltransferase_{hmb}, N-cadherin_{hm}, neural cell adhesion molecule_{hmb}, neurotrophin-3_{hm}, NF- κ B_{hm}, N-myc_{hm}, nucleolar protein N038_{hm}, nucleolin_{hm}, ornithine decarboxylase_{hmb}, p53 cellular tumour antigen_{hmb}, S6 II kinase_{hm}, SPARC_{hmb}, transcription factor Eryf-1_{hm}, TGF- β 2_{hm}, TGF- β 3_{hm}, transglutaminase_{hmb}, tyrosine phosphatase_{hm}, vimentin_{hmb} (for all genes, sequences of *G. gallus* and an outgroup, usually *X. laevis* or a paralogous sequence, also were used).

research has generated a large number of gene sequences for representatives of several orders of birds and mammals. This now allows us to derive a single average estimate of divergence time from many independent (single gene) estimates, thus reducing potential biases that may exist with individual genes, and increasing the accuracy of the time estimate.

An accurate calibration point is also required for a reliable estimate of divergence time from molecular data. Typically, the first occurrence of two taxa in the fossil record is used as a minimum estimate of their time of divergence. However, that may be a considerable underestimate (> 50%) because of sampling biases⁶. The fossil record of birds and mammals is particularly prone to such biases because of the long time span between the earliest fossils (150 million years (Myr) for birds, 220 Myr for

mammals) and the first appearances of the modern orders (mostly 55–65 Myr)⁴. Therefore, an internal calibration based on the fossil record of the modern orders themselves may lead to a large bias in the divergence times estimated. To avoid this, we have chosen the relatively well-constrained fossil divergence time between the ancestors of birds (diapsid reptiles) and mammals (synapsid reptiles), 310 Myr, as an external calibration point⁴. The first appearance of amniotes at 335 Myr (ref. 11) and of tetrapods at 370 Myr (ref. 12) suggests that a divergence time of birds and mammals much earlier than 310 Myr is unlikely.

All of our molecular estimates of divergence times for avian and mammalian orders are Mesozoic rather than Cenozoic, and are considerably older than divergence times suggested by fossil evidence (Table 1; Fig. 1). Some previous estimates of divergence



times based on immunological distances and sequence data also pre-date the K/T boundary⁷⁻¹⁰. However, unlike those studies, we have tested for rate constancy both among orders and between the lineages used for calibration, used an external (bird-mammal) rather than an internal calibration, and based our divergence times exclusively on a large number of constant-rate genes.

We suggest that the fragmentation of emergent land areas during the Cretaceous, not the sudden availability of ecological niches following the K/T extinction event, was the mechanism responsible for the diversification of avian and mammalian orders. When plate tectonics and sea level are considered together, all of the major continental breakup events during the last 250 Myr occurred in the Mesozoic, and mainly during the Cretaceous (Figs 1c and 2). The timing of these events corresponds closely to the molecular time estimates for divergences of the orders (Fig. 1b). The tectonic breakup of Pangaea has been cited as a factor in the historical biogeography of some Mesozoic organisms, including birds and mammals^{9,13}. However, few data on times of divergence from constant-rate genes have been available to test such a hypothesis. Moreover, comparisons have not been made between ordinal divergence times of multiple groups such as birds and mammals. The continental breakup hypothesis (Fig. 2) is compatible with the extensive speciation that occurred within orders following the sudden availability of niches in the early Tertiary period.

These early molecular dates for divergences among the orders of birds and mammals indicate an unusually strong bias in the fossil record against Middle to Late Cretaceous fossils. Other groups of terrestrial vertebrates, including dinosaurs, also show a mid-Cretaceous decline in diversity¹⁴, but not as pronounced. Besides the sampling bias already noted⁶, the highest sea levels in the Mesozoic and Cenozoic occurred in the Middle to Late Cretaceous, resulting in about 25% less emergent land area than exists at present (Fig. 1d). This would have proportionately reduced the area available for the deposition of terrestrial fossils.

The general concordance between ordinal diversification and Earth history for birds and mammals predicts that similar patterns may be seen in other terrestrial organisms. Although the relationships and times of divergence of turtle, lizard and snake families are not yet well established^{4,15}, Mesozoic continental fragmenta-

TABLE 1 Molecular time estimates for the divergence of avian and mammalian orders*

Comparisons	No. of genes		Total length†	Time‡ (Myr)	Evolutionary rate ($\times 10^{-4}$ per site per Myr)
	Total	Constant rate			
Birds					
Nuclear genes§	34	19	2,829	97 $\pm$ 12	1.9–13.1
Mitochondrial genes					
rRNA	2	2	1,267	68–131	2.8
Protein	3	0			
Mammals					
Nuclear genes					
Primate–Rodent	48	24	10,051	95 $\pm$ 7	0.8–8.5
Primate–Artiodactyl	22	17	6,573	90 $\pm$ 8	0.4–10.0
Rodent–Artiodactyl	20	12	4,497	113 $\pm$ 9	1.0–10.2
Mitochondrial genes					
rRNA	2	2	2,171	99–184	4.0
Protein	13	0			

Nuclear genes. Complete amino-acid sequences (≥ 100 residues) of nuclear genes were obtained (HOVERGEN¹⁶, release 10; ENTREZ, release 18). Avian taxa included a struthioniform (*Struthio camelus*), galliform (*Gallus gallus*), anseriform (*Anas platyrhynchos* or *Cairina moschata*) and columbiform (*Columba livia*); corresponding sequences of a mammal (for calibration) and a more distantly related taxon (usually *Xenopus laevis* or a paralogous sequence, for root) were included. Mammalian taxa included a primate (*Homo sapiens*), rodent (*Mus musculus*) and artiodactyl (*Bos taurus*); relationships among these orders generally have not been well established¹⁹, although molecular evidence has favoured a basal position for rodents¹⁰. For mammalian analyses, each comparison included data from a bird (*G. gallus*) for calibration and an outgroup (usually *X. laevis* or a paralogous sequence) for root. Sequences of individual genes (or gene families) were aligned²⁰ and orthology determined by phylogenetic analysis using MEGA²¹. Sites with alignment gaps were removed and constancy of rate between mammalian orders and between *G. gallus* and the mammals was tested (5% level)²². Pairwise distances were estimated by use of a gamma correction²³ with shape parameter (α) of 2. For each gene, average pairwise distance between *G. gallus* and the two mammalian sequences was divided by 620 (2×310 Myr) to obtain evolutionary rate, and the pairwise distance between mammalian orders was converted to time using this estimate of rate. Ordinal divergences were estimated with the Poisson correction distance and a different gamma distance ($\alpha = 1$); resulting estimates were within $\pm 5\%$ of those in the table. Assuming a basal position of rodents, the average time estimate (nuclear genes) of the divergence of the rodent lineage from the primate–artiodactyl lineage is 104 Myr. The timing of avian ordinal divergences was accomplished similarly, although fewer sequences ($\geq$ approximately 100 residues) were available, so all pairwise comparisons for nuclear genes were pooled to estimate means. S.e.m. was computed by dividing the standard deviation by the square root of the number of different genes to account for non-independence in multiple comparisons. **Mitochondrial genes.** For avian comparisons, portions of the two rRNA genes were analysed by the linearized tree method using a gamma correction distance ($\alpha = 1.7$, transversional distance under Kimura's model²⁴) for representatives of 7 avian orders: galliform (*G. gallus*), anseriform (*Anas platyrhynchos*), struthioniform (*Rhea americana*), psittaciform (*Melopsittacus undulatus*), columbiform (*Columba livia*), cuculiform (*Cuculus pallidus*), and ciconiiform (*Ciconia nigra*). The amino-acid sequences of ATP6, ATP8 and COXII from *G. gallus* and *A. platyrhynchos*²⁴ also were analysed using a gamma correction distance ($\alpha = 1.4$, Poisson model²⁵). In both cases, an artiodactyl (*B. taurus*) and carnivore (*Phoca vitulina*) were used for calibration, and a frog (*X. laevis*) was included for root. For mammalian comparisons, the taxa²⁵ are: an artiodactyl (*B. taurus*), carnivore (*P. vitulina*), cetacean (*Balaenoptera physalus*), perissodactyl (*Equus caballus*), primate (*H. sapiens*), rodent (*M. musculus*), a galliform bird (*G. gallus*, for calibration), and a frog (*X. laevis*, for root). The linearized tree method²⁶ was used with the gamma distance ($\alpha = 1.4$ for protein genes, $\alpha = 1.7$ for rRNA genes)^{21,27}. In both avian and mammalian analyses using linearized tree methods, rate constancy was rejected for protein genes but not for rRNA genes. However, the likelihood ratio tests²⁸ (using Kimura's model with $R = 2$) rejected rate constancy in rRNA genes as well. Therefore dates obtained by the linearized tree method are not shown in Fig. 1.

* Nuclear genes were analysed individually, and mitochondrial genes were analysed tandemly as two datasets (protein and rRNA genes). Total sequence lengths, times of divergence, and evolutionary rates are shown only for genes in which constancy of evolutionary rate was not rejected.

† Number of aligned amino-acid residues (proteins) or nucleotides (rRNA) for constant-rate genes.

‡ Mean time of divergence (± 1 s.e.) as computed from the time estimates for comparisons of nuclear genes. For mitochondrial genes, the range of time is given for all ordinal divergences estimated in the linearized trees.

§ Based on pairwise comparisons of galliform, anseriform, columbiform and struthioniform birds.

|| Constant rate was rejected in likelihood ratio tests but not in linearized tree tests.

tion has been implicated in dinosaur biogeography¹⁶. Similarly, recent DNA sequence evidence about frog relationships¹⁷ identified two major groups of families associated with Laurasia (Archaeobatrachia) and Gondwana (Neobatrachia); the two major neobatrachian groups are associated with South America (Bufonoidea) and Africa (Ranoidea). These observations conform to the hypothesis of continental breakup and diversification. Major radiations of marine invertebrates in the mid-Cretaceous also have been causally linked to the subdivision of continents by epicontinental seas¹³.

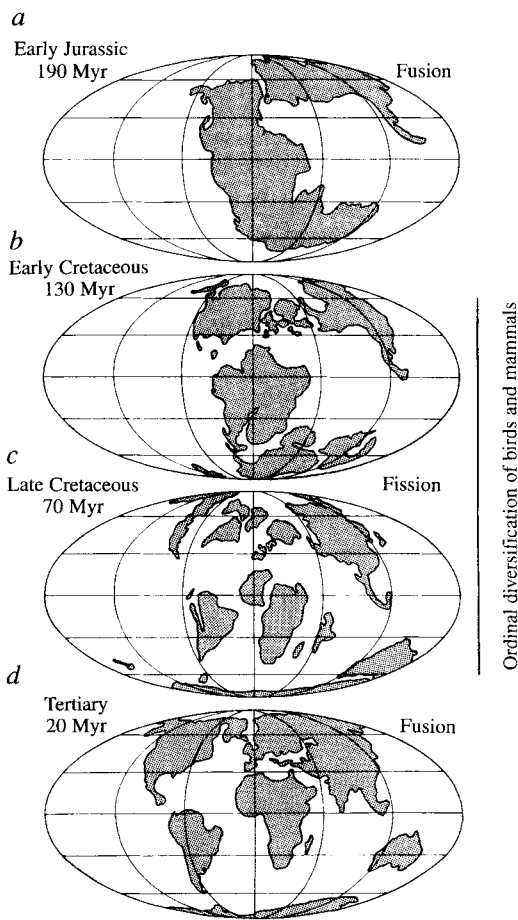


FIG. 2 Breakup (fission) and fusion of emergent land areas in the Mesozoic and Cenozoic as determined by plate-tectonic reconstructions and paleocoastline data (modified from ref. 30). *a*, Early Jurassic (190 Myr), with 2 large continents. *b*, Early Cretaceous (130 Myr), with 7 continents ($> 3 \times 10^6$ km²). *c*, Late Cretaceous (70 Myr), with 11 continents. *d*, Mid-Tertiary (20 Myr), with 5 continents; subsequent fusion of North and South America, and Africa with Eurasia, led to the present configuration of three isolated emergent land masses. The orders of mammals and birds diverged during the Cretaceous (for example, *b* and *c*) at the time when emergent land areas were undergoing breakup.

If continental breakup were an important mechanism for ordinal diversification in birds and mammals, then it might be expected that distributional data of living fossil taxa should help in biogeographic reconstruction. Although some orders can be associated with specific palaeolandmasses⁹, most now occur on multiple continents and their original distributions have not been established. This is not surprising considering that a large amount of continental fusion, allowing migration, already had occurred by the Early Tertiary when the fossils of most modern orders first appeared (Fig. 1*a, c*). As larger numbers of constant-rate genes for a diversity of taxa become available, it should be possible more accurately to associate ordinal divergences with specific continental breakup events. □

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1. Romer, A. S. *Vertebrate Paleontology* (Univ. Chicago Press, 1966).
2. Strickberger, M. W. *Evolution* (Jones and Bartlett, Boston, MA, 1995).
3. Feduccia, A. *Science* **267**, 637–638 (1995).
4. Benton, M. J. (ed.) *The Fossil Record Vol. 2* (Chapman and Hall, New York, 1993).
5. Chiappe, L. M. *Nature* **378**, 349–355 (1995).
6. Martin, R. D. *Nature* **363**, 223–234 (1993).
7. Prager, E. M., Brush, A. H., Nolan, R. A., Nakanishi, M. & Wilson, A. C. *J. molec. Evol.* **3**, 243–262 (1974).
8. Li, W.-H., Gouy, M., Sharp, P. M., O'Huigin, C. & Yang, Y.-W. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6703–6707 (1990).
9. Easteal, S., Collet, C. & Betty, D. *The Mammalian Molecular Clock* (Landes, Austin, TX, 1995).

10. Janke, A., Feldmaier-Fuchs, G., Thomas, W. K., von Haeseler, A. & Pääbo, S. *Genetics* **137**, 243–256 (1994).
11. Smithson, T. R. *Nature* **342**, 676–678 (1989).
12. Ahlberg, P. E. & Milner, A. R. *Nature* **368**, 507–514 (1994).
13. Hallam, A. *An Outline of Phanerozoic Biogeography* (Oxford Univ. Press, 1994).
14. Padian, K. & Clemens, W. A. in *Phanerozoic Diversity Patterns* (ed. Valentine, J. W.) 41–96 (Princeton Univ. Press, NJ, 1985).
15. Zug, G. R. *Herpetology* (Academic, San Diego, CA, 1993).
16. Russell, D. A. *Hist. Biol.* **10**, 3–12 (1995).
17. Hay, J. M., Ruvinsky, I., Hedges, S. B. & Maxson, L. R. *Molec. Biol. Evol.* **12**, 928–937 (1995).
18. Duret, L., Mouchiroud, D. & Gouy, M. *Nucleic Acids Res.* **22**, 2360–2365 (1994).
19. Novacek, M. *Nature* **356**, 121–125 (1992).
20. Higgins, D. G., Bleasby, A. J. & Fuchs, R. *Comput. Appl. Biosci.* **8**, 189–191 (1992).
21. Kumar, S., Tamura, K. & Nei, M. *Molecular Evolutionary Genetics Analysis* version 1.01 (Pennsylvania State University, 1993).
22. Tajima, F. *Genetics* **135**, 599–607 (1993).
23. Ota, T. & Nei, M. *J. molec. Evol.* **38**, 642–643 (1994).
24. Ramirez, V., Savoie, P. & Morais, R. J. *molec. Evol.* **37**, 296–310 (1993).
25. Xu, X. & Arnason, U. *Gene* **148**, 357–362 (1994).
26. Takezaki, N., Rzhetsky, A. & Nei, M. *Molec. Biol. Evol.* **12**, 823–833 (1995).
27. Hedges, S. B. & Sibley, C. G. *Proc. natn. Acad. Sci. U.S.A.* **91**, 9861–9865 (1994).
28. Felsenstein, J. *Phylogenetic Inference Package (PHYLIP)*, Version 3.5 (University of Washington, Seattle, 1993).
29. Sibley, C. & Ahlquist, J. *Phylogeny and Classification of Birds* (Yale Univ. Press, New Haven, CT, 1990).
30. Smith, A. G., Smith, D. G. & Funnell, B. M. *Atlas of Mesozoic and Cenozoic Coastlines* (Cambridge Univ. Press, 1994).

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CORRESPONDENCE and requests for materials should be addressed to S.B.H. (e-mail: sbh1@psuvm.psu.edu). Accession numbers for these sequences can be obtained from the Nature World-Wide Web site. (<http://www.nature.com>).

Correlation between male song repertoire, extra-pair paternity and offspring survival in the great reed warbler

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In many birds, females copulate with males other than their social mate, resulting in extra-pair fertilizations (EPFs)^{1–7}. It is still unknown, however, why females seek EPFs^{7,8}. In one study, males that accounted for most EPFs had higher survival⁶, but neither the characteristics revealing male quality nor the benefits accruing to females selecting attractive males were identified. Great reed warblers, *Acrocephalus arundinaceus*, are socially polygynous, and females base their mate choice on territory quality⁹ and song-repertoire size¹⁰, both of which predict harem size and reproductive success^{11,12}. By DNA fingerprinting¹³, we demonstrate that female great reed warblers obtain EPFs from neighbouring males with larger song repertoires than their social mate. In addition, the relative post-fledging survival of offspring was positively correlated with their genetical fathers' song repertoire size. These data support the hypothesis that females, by engaging in extra-pair fertilizations, seek genetic benefits for their offspring^{7,8}.

We studied a population of individually colour-ringed great reed warblers, a long-distance migrant with sexually monomorphic cryptic plumage, at lake Kvismaren, Sweden^{9,12,14–18} from 1987 to 1993. All territories were visited daily throughout the breeding season (May–July). In this population, a substantial proportion of the returning migrants settle in their natal area¹⁸. Polygynous males (40% of territorial males^{12,14}) attract females sequentially about every 8 days¹⁶. Males have a loud mate-attraction

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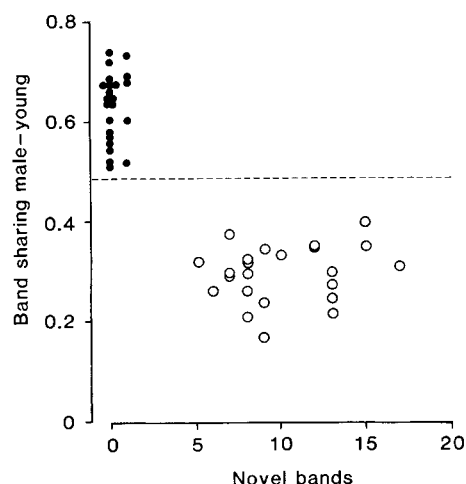


FIG. 1 DNA band-sharing¹ value and number of novel bands⁵ for each of the 23 great reed warbler young that were sired by extra-pair males compared with their putative father (social male, open circles) and their assigned genetical father (EPF-male, filled circles). The broken line denotes the lower 99% confidence limit for band sharing between a parent and its genetical offspring¹⁵. DNA fingerprint bands, from genomic DNA digested with *AluI* and hybridized with Jeffreys 33.15 probe¹³, were scored in the approximate size range of 2.5–23 kb (see ref. 15 for detailed descriptions of DNA-fingerprinting methods). Band sharing between parents and genetical offspring was on average 0.66 (s.d. = 0.07) (ref. 15). For each young we also measured the number of novel (mismatched) bands, that is, bands possessed by the young that were not present in either of the putative parents¹⁵. Extra-pair paternity (the putative female was in all cases the genetical mother¹⁵) for each young with novel bands was examined on new gels that included the potential fathers¹⁵. DNA fingerprint bands show low frequency of linkage in the great reed warbler, and can therefore be considered to be independently inherited³⁰. DNA fingerprinting was performed on 14, 35, 28, 29 and 24 broods from the years 1987–1991, respectively. This data set includes > 95% of all broods in our study area from which the young survived until fledging. In addition, we performed DNA fingerprinting on 35 broods (71% of all broods with fledglings) in 1992–1993. In total, the data set consisted of 678 young (162 broods). We took DNA samples from all males that held territories in the study area in 1987–1993. Male floaters are uncommon in this population, and most of those that prevail take up territories in the study area after a few days.

TABLE 1 Paired comparisons of characteristics of social males and EPF-males in the 10 broods (indexed 1–10) containing extra-pair young

	Male status*	Nest (year) exposed to cuckoldry										E > S†
		1 (1988)	2 (1988)	3 (1989)	4 (1989)	5 (1989)	6 (1991)	7 (1991)	8 (1992)	9 (1993)	10 (1993)	
Song-repertoire size	S	34	38	32	36	32	42	43	27	37	27	10‡
	E	38	40	36	42	36	43	45	41	43	37	
	E – S	+4	+2	+4	+6	+4	+1	+2	+14	+6	+10	
Age (years)	S	1	2	2	3	1	1	5	2	3	1	8, NS§
	E	3	3	1	4	3	4	3	6	4	3	
	E – S	+2	+1	–1	+1	+2	+3	–2	+4	+1	+2	
Tarsus length	S	33.2	34.1	33.7	33.3	33.7	34.1	34.1	34.8	34.0	33.0	4, NS
	E	34.1	34.4	33.3	34.6	33.3	33.1	33.7	34.1	33.6	34.0	
	E – S	+0.9	+0.3	–0.4	+1.3	–0.4	–1.0	–0.4	–0.7	–0.4	+1.0	
Territory attractiveness	S	13	13	4	1	11	6	9	7	2	9	5, NS
	E	13	3	4	4	1	13	6	2	4	2	
	E – S	0	–10	0	+3	–10	+7	–3	–5	+2	–7	
Total harem size	S	2	1	2	3	1	1	3	3	3	1	3, NS
	E	1	2	2	2	3	1	3	3	1	3	
	E – S	–1	+1	0	–1	+2	0	0	0	–2	+2	
Territory occupation date	S	20	14	15	1	55	17	7	16	2	19	6, NS
	E	14	11	15	12	1	14	17	3	2	2	
	E – S	–6	–3	0	+11	–54	–3	+10	–13	0	–17	

Recordings of song repertoire were made with a Sony TC-DM 5 cassette tape recorder and a parabola (Telenga) with microphone, at a distance of 10–50 m from the singing male. We recorded 4–5 min of the loud mate-attraction song^{12,14,29} from ≥ 95% of all territorial males in the study area in 1987–1993. Sonograms were made on a Kay Elemetrics DSP Sona-graph, model 5500 or using the software program Canary 1.2 (Bioacoustics Lab, Cornell University, USA) with the settings: frequency range, 0–8 kHz; transform size, 128 points. Printouts were analysed visually without knowing from which male each sonogram originated. We estimated song repertoire size on the basis of different syllables (different sounds), a method used in several species of *Acrocephalus*, including the great reed warbler^{11,29}. Great reed warblers use a rather limited number of syllables sung in different combinations to form a large number of different song 'strophes'. At least 150 syllable switches (switches between short series of repeated syllables) were analysed for each male, corresponding to 18–22 song strophes. Total repertoire size is the cumulative number of different syllables over 150 syllable switches. Cumulative song repertoire size in the great reed warbler levels off after 10–12 song strophes^{11,29}. We found that repertoire size averaged 36 different syllables (s.d. = 5.0, range 23–45) per 150 syllable switches. For ageing, previously unringed birds were classified as either 1 or 2 years old, on the basis of tongue spots, and eye and tarsus colour^{12,16}. Tarsus length, to the nearest 0.1 mm, was measured from the bent foot to the bent joint between the tibia and the tarsus, using callipers¹². Territory attractiveness is based on the sequence by which the territory was occupied in the two years flanking the focal year. Thus territory attractiveness for year_x is the mean of the order in which the actual territory became occupied in year_{x-1} and year_{x+1} (see ref. 16 for more details). In our study population, each territory's attractiveness is highly correlated between years¹⁸. Harem size is the number of females that were nesting in the male's territory during the focal breeding season. Territory occupation date is the first day a male was heard singing in a territory; day 1 is 1 May.

* S, social male; E, EPF-male; E – S = value for EPF-male minus value for social male.

† Number of cases when the EPF-male's value was higher, larger or earlier than the social male's value.

‡ Binomial test, two-tailed, $P = 0.0020$. With sequential Bonferroni correction, P_{crit} is $0.05/6 = 0.0083$. The mean ($\pm$ s.e.) song repertoire size was 40.1 ± 1.0 for EPF-males, and 34.8 ± 1.7 for the cuckolded social males.

§ Binomial test, two-tailed, $P = 0.11$. With sequential Bonferroni correction, P_{crit} is $0.05/5 = 0.01$.

|| Not significant ($P > 0.15$).

song, but do not sing it when mate guarding or feeding young, and so do not attract additional females at these times^{12,14}.

We can unambiguously define a female's social mate as the male that guarded the female and attended her at a nest in his territory. No female that obtained EPFs (EPF-female) changed her social mate during the fertile period¹⁵. EPF-males, with which EPF-females obtained EPFs, sired 23 extra-pair young (EPY) (3% of 678 fledglings) in 10 out of 162 broods (6%) in 1987–1993. The genetical fathers of all EPY were identified (Fig. 1), and in each of the ten broods only one male sired the EPY.

Measures of male song-repertoire size, age, body size, territory attractiveness and territory occupation date were obtained for all territorial males in 1987–1991 (complete sets of measurements were not obtained in 1992–1993). In a multivariate analysis, only song-repertoire size predicted the likelihood that males would get EPFs (logistic regression¹⁹, $G = 6.4$, $P = 0.025$, $N = 94$ male breeding seasons). This result was confirmed in a paired comparison of each EPF-male and the respective cuckolded pair-male (the female's social mate) (Table 1). In all 10 cases, the EPF-males had the larger song repertoire (binomial test, $P = 0.0020$).

EPF-males were always neighbours, residing within 120 m of the nest containing their EPY. Within this distance, a singing male neighbour was available to the fertile female in 33% of the cases (45 of 130 broods in 1987–1991). In 50% of these cases (23 of 45), the neighbour was singing with a larger repertoire than the social mate. EPY occurred in 30% (7 of 23) of the latter females' broods, but no EPY occurred when neighbours had smaller repertoires (0 of 22) (Fisher test, $P = 0.009$). Thus the low frequency of EPFs in the great reed warbler may result from few fertile females having opportunities for extra-pair copulations with a preferred male (18% of 130 broods), that is, a close neighbour singing with a large repertoire. Our data do not imply that females passively accepted

EPFs. If this had been the case, cessation of mate guarding during the female's fertile period should have increased the probability of EPFs, but no such relationship occurred¹⁵.

There was a positive correlation between the males' lifetime number of sired fledglings and recruits (offspring returning as adults) ($P < 0.001$; Fig. 2a). The residual values of this regression (Fig. 2a) describe the variation in lifetime number of recruits that is not explained by lifetime number of fledglings. This measure of relative post-fledging survival of genetical offspring was positively correlated with male standardized song repertoire size (repertoire size statistically compensated for age; see Fig. 2 legend) ($P = 0.028$, Fig. 2b). This relationship is not likely to be a consequence of parental efforts, such as males with large repertoires providing better care for the offspring, or females investing more in offspring sired by such males; there was no correlation ($r = 0.016$, $N = 116$ broods, NS) between male standardized song-repertoire size and the mean body mass of the fledglings (a key factor for post-fledging survival in passerines²⁰, including the great reed warbler (D.H., S.B. and T.v.S., unpublished results)).

The relationship in Fig. 2b was not biased owing to differences in natal dispersal of offspring sired by males with different song repertoires. There was no difference in repertoire size between males whose recruiting offspring all returned to their natal area ($N = 29$ males) and those where at least one offspring recruited outside the natal area (> 15 km away; $N = 9$ males) (logistic regression, $G = 1.0$, NS). Thus all available data suggest that male song repertoire was the sole predictor of relative post-fledging survival.

There was a tendency ($P = 0.11$) for EPF-males to be older than pair-males (Table 1). Because song repertoire size is positively related to age (see Fig. 2 legend), it may be that females select older males by their song repertoire²¹. However, male age

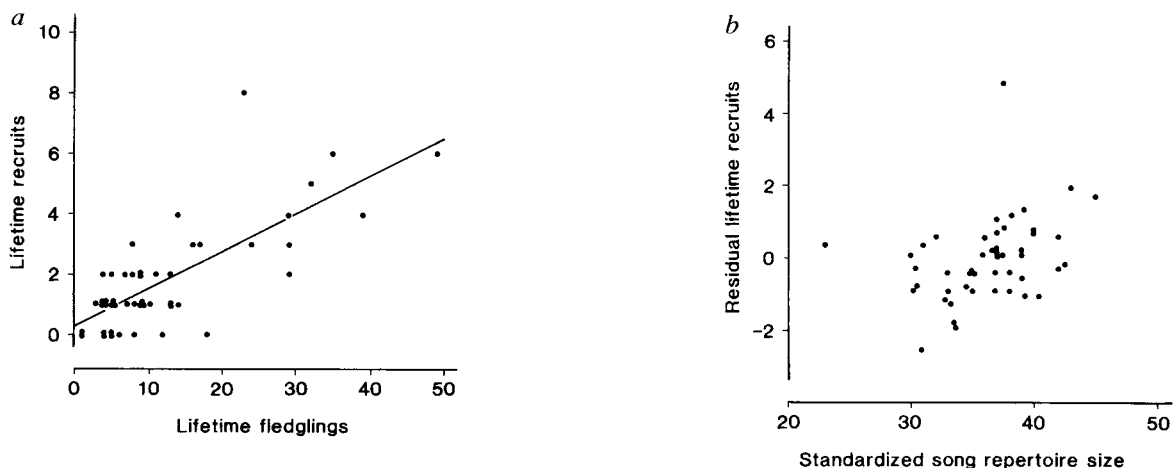


FIG. 2 a, Regression of number of lifetime recruits per male on number of lifetime fledglings ($r = 0.76$, $N = 48$, $P < 0.001$). Recruits are fledglings hatched in the study area that returned there, or to other marshes in south central Sweden (dispersing 15–150 km) the next breeding season or later. Only males that sired at least one fledgling during 1987–1991 are included in the analysis. b, Correlation between standardized song repertoire size and relative postfledging survival, that is, the residual values from the regression in Fig. 2a ($r = 0.32$, $N = 48$, $P < 0.028$). If the two outliers are excluded there is an even stronger positive relationship between standardized song repertoire size and relative postfledging survival ($r = 0.46$, $N = 46$, $P < 0.0014$). Relative postfledging survival was not correlated with male life span, tarsus length, mean territory attractiveness or mean territory occupation date (lifetime data for 48 males). There was no correlation between standardized song repertoire size and number of lifetime fledglings¹². A multiple regression analysis based on annual data pooled for the five years (1987–1991; $N = 77$ male breeding seasons) revealed a significant correlation between the annual value of the males' song repertoire size and relative postfledging survival. In this analysis (data not

shown), male song repertoire size (partial $r = 0.25$, $P = 0.029$), harem size (partial $r = -0.26$, $P = 0.021$) and number of fledglings (partial $r = 0.58$, $P = 0.001$) were significantly correlated with the number of recruits when the effects of the other two variables were partialled out (multiple $r = 0.61$, $P = 0.001$). When the effects of repertoire size, harem size and number of fledglings were kept constant, neither territory attractiveness (partial $r = -0.16$, NS) nor territory occupation date (partial $r = -0.03$, NS) had any effect on number of recruits. Male song repertoire size was positively related to age ($F_{(3,103)} = 11.5$, $P < 0.001$; average number of different syllables for 1-year-old males, $\bar{x} = 32.00$, $N = 29$; 2 years, $\bar{x} = 35.95$, $N = 37$; 3 years, $\bar{x} = 38.08$, $N = 24$; 4 years, $\bar{x} = 38.35$, $N = 17$). The average differences between ages were used to compute the repertoire size of each male adjusted to an age of 2 years using the formula $MRS_i + (35.95 - ARS_i)$, where MRS_i is a male's repertoire size when i years old, and ARS_i is the average repertoire size of all males at the age of i years. Standardized song repertoire size is a male's adjusted repertoire size averaged for all ages from which it was computed.

did not correlate with annual production of recruits ($P = 0.35$), nor with relative post-fledging survival of offspring ($P = 0.87$) ($N = 77$ male breeding seasons).

If female mate choice provides direct benefits, it can be difficult to evaluate the importance of 'good genes'²². Indeed, two direct benefits, paternal care and territory attractiveness (defined in Table 1), were both positively related to annual production of fledglings^{16,18}; however, EPF-males did not contribute any of these resources to the EPF-female¹⁵, and neither correlated with relative post-fledging survival^{12,16} (see Fig. 2 legend). Nor was there any correlation between standardized song-repertoire size and proportion of eggs hatching (counting all a male's clutches without EPY; $r = 0.06$, $N = 45$, NS). Thus, although direct benefits seem to determine the number of fledged young^{12,16}, the relative post-

fledging survival of offspring seemed to be mainly affected by indirect (genetic) benefits obtained from males with large song repertoires.

The idea that females discriminate among males to obtain indirect (genetic) benefits for their offspring has been questioned²². Although females' attraction to ornamented males is well documented^{23–25}, few studies have indicated that male ornamentation may correlate with offspring survival^{26–28}. Here we demonstrate the importance of indirect (genetic) benefits in female mate choice, because we have found (1) that females seek EPFs from males with large song repertoires seemingly without obtaining any direct benefits, and (2) that the preferred male character (large song repertoire) predicts the relative post-fledging survival of offspring. □

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1. Wetton, J. H., Carter, R. E., Parkin, D. T. & Walters, D. *Nature* **327**, 147–149 (1987).
2. Burke, T. & Bruford, M. W. *Nature* **327**, 149–152 (1987).
3. Gibbs, H. L. *et al. Science* **250**, 1394–1397 (1990).
4. Westneat, D. F. *Behav. Ecol. Sociobiol.* **27**, 67–76 (1990).
5. Westneat, D. F. *Behav. Ecol.* **4**, 49–60 (1993).
6. Kempenaers, B. *et al. Nature* **357**, 494–496 (1992).
7. Birkhead, T. R. & Møller, A. P. *Sperm Competition in Birds: Evolutionary Causes and Consequences* (Academic, Orlando, 1992).
8. Westneat, D. F., Sherman, P. W. & Morton, M. L. *Curr. Orn.* **7**, 331–369 (1990).
9. Bensch, S. & Hasselquist, D. *Anim. Behav.* **44**, 301–311 (1992).
10. Catchpole, C. K., Leisler, B. & Dittami, J. *Ethology* **73**, 69–77 (1986).
11. Catchpole, C. K. *Behav. Ecol. Sociobiol.* **19**, 439–445 (1986).
12. Hasselquist, D. Thesis, Lund University, Sweden (1994).
13. Jeffreys, A. J., Wilson, V. & Thein, S. L. *Nature* **314**, 67–73 (1985).
14. Hasselquist, D. & Bensch, S. *Behav. Ecol. Sociobiol.* **28**, 187–193 (1991).
15. Hasselquist, D., Bensch, S. & von Schantz, T. *Behav. Ecol.* **6**, 27–38 (1995).
16. Bensch, S. thesis, Lund Univ. Sweden (1993).
17. Bensch, S. & Hasselquist, D. *Am. Nat.* **138**, 1297–1306 (1991).
18. Bensch, S. & Hasselquist, D. *J. Anim. Ecol.* **60**, 857–871 (1991).
19. Steinberg, D. & Colla, P. *LOGIT: A Supplementary Module for SYSTAT*. (SYSTAT, Evanston, IL, 1991).
20. Magrath, R. D. *J. Anim. Ecol.* **60**, 335–351 (1991).
21. Wetton, J. H., Burke, T., Parkin, D. T. & Cairns, E. *Proc. R. Soc. Lond. B* **260**, 91–98 (1995).

22. Kirkpatrick, M. & Ryan, M. J. *Nature* **350**, 33–38 (1991).
23. Andersson, M. *Nature* **299**, 818–820 (1982).
24. von Schantz, T. *et al. Nature* **337**, 166–169 (1989).
25. Møller, A. P. *Nature* **332**, 640–642 (1988).
26. Norris, K. *Nature* **362**, 537–539 (1993).
27. von Schantz, T., Grähn, M. & Göransson, G. *Am. Nat.* **144**, 510–527 (1994).
28. Petrie, M. *Nature* **371**, 598–599 (1994).
29. Catchpole, C. K. *Anim. Behav.* **31**, 1217–1225 (1983).
30. Bensch, S., Hasselquist, D. & von Schantz, T. *Evolution* **48**, 317–326 (1994).

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Sexually antagonistic male adaptation triggered by experimental arrest of female evolution

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EACH sex is part of the environment of the other sex. This may lead to perpetual coevolution between the sexes, when adaptation by one sex reduces fitness of the other. Indirect evidence comes from experiments with *Drosophila melanogaster* indicating that seminal fluid reduces the competitive ability of sperm from other males, thereby increasing male fitness^{1,2}. It also reduces a female's propensity to remate and increases her egg-laying rate³. In contrast to these benefits to males, seminal fluid has substantial toxic side effects in females, with increasing quantity leading to decreasing female survival^{4,5}. Here I show that when female *D. melanogaster* are experimentally prevented from coevolving with males, males rapidly adapt to the static female phenotype. This male adaptation leads to a reduction in female survivorship, which is mediated by an increased rate of remating and increased toxicity of seminal fluid.

To allow males to evolve while preventing females from counter-evolving, two large populations were constructed: a non-responding stock that donated target females unidirectionally to the experimental adapting-male line (Fig. 1). The target females could not evolve in response to the experimental males because they were

taken anew each generation from the non-responding stock. Males could evolve, however, in response to the phenotype of target females. By using artificial selection on marked chromosomes, 99% of the genome derived from non-responding females was cycled out of the adapting-male line after being used for a single generation (see Fig. 1 for method). Thus non-responding females were essentially static targets for the adaptation of virtually an entire haploid genome of the adapting-male line.

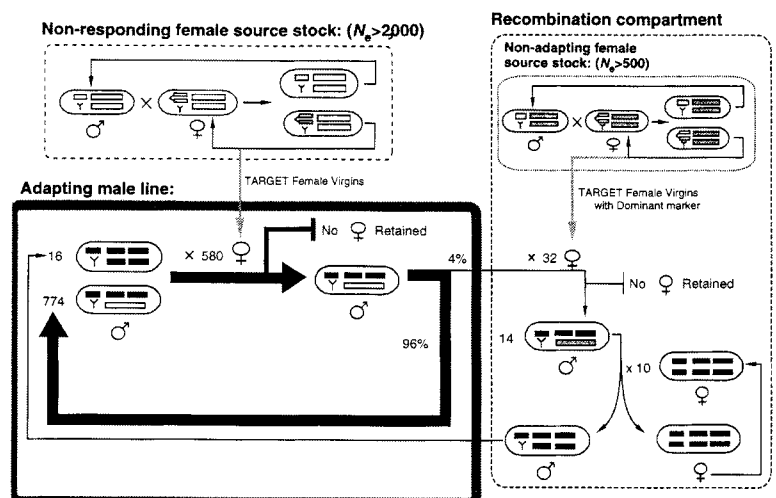
Artificial selection and the absence of recombination in male *Drosophila* prevented recombination in the adapting-male line. Theory indicates that lack of recombination substantially impedes adaptation, owing to slower accumulation of beneficial alleles and faster accumulation of deleterious mutations^{6–10}. Small amounts of recombination, however, eliminate most of this cost. Therefore, to speed the rate of adaptation despite a minor reduction in the strength of selection, a small fraction (4%) of the genomic haplotypes were passed through a series of crosses that recombined adapting-male genomes (Fig. 1, recombination compartment).

The experiment continued for 41 generations and comprised two replicates (A and B), each with an experimental adapting-male line (EA or EB) and a control line (CA or CB). The control-line males did not contain a marked autosomal translocation that was present in the experimental lines. To insert this translocation into the control lines, beginning in generation 29, samples of males from each control line were crossed with target females from the non-responding stock to produce sons that had the same genetic background as the experimental males. These males were then subjected to the same procedure as the experimental males for one generation to produce sons (CA' and CB', used in fitness assays) before being discarded.

After 30 generations there was evidence for substantial adaptation of the experimental males to the target females (Fig. 2). The observed 24% average increase in net fitness is associated with

FIG. 1 A combination of special chromosomes and artificial selection allows males of the adapting-male line to adapt to target females from the non-responding female source stock but not vice versa. The Y chromosome is depicted by 'Y' and others by rectangles; chromosomes I (X), II and III are at left, middle and right positions respectively. The unmanipulated dot chromosome IV (1% of genome) is not shown. Connected X chromosomes depict the compound X C(1)DX y f and double-length rectangles depict the translocation T(2;3)-rdgC^{COE}st in ri p^p (flies were used before the onset of expression of the *rdgC* gene). The stippled translocations carry the additional dominant marker *bw^p*. Genotypes produced in a cross but not shown were removed while sexually immature by means of artificial selection.

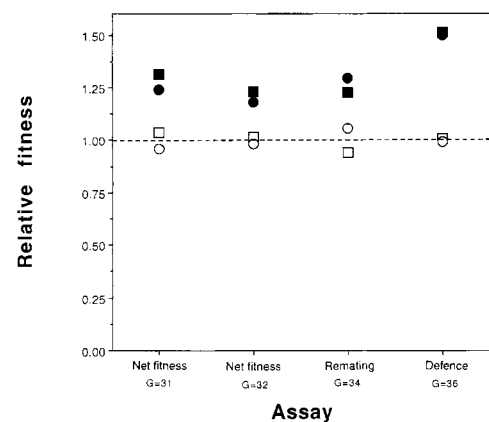
METHODS. To begin each of the two experiments (EA and CA or EB and CB), a sample of 1,000 mated females was taken from a large outbred population (LH, established two years earlier by L. Harshman from 400 mated females collected in central California and maintained at 25 °C with $N_e > 5,000$ on a two-week generation time). From their offspring, 250 mated females and 600 males were collected and labelled 'founders'. The founder males were mass mated to 500 target virgin females (here and throughout, virgin females used in matings were 2–3 days old) to produce the original 774 translocation heterozygous males of the adapting male line. The original 14 males in the recombination compartment came from a parallel cross of 40 additional founder males with 30 target females carrying the dominant marker *bw^p*. The remaining 16 males with normal karyotypes in the adapting-male line came from offspring of the 250 mated founder females, as did the 10 females in the recombination compartment. The purpose of the recombination compartment was to extract X, II and III genomic haplotypes from the adapting-male line, recombine them, and then return them to the adapting-male line. Throughout the experiment, the adapting-male line flies were mixed and then divided into 18 vials (2.5 cm × 17.5 cm tall), each containing 43 males and



32 virgin target females. In each of four of these vials, an additional four males were added from the recombination compartment. The males and females were mass transferred daily to fresh vials, and offspring from the first two transfers were used to propagate each successive generation. The control population, consisting of 203 males combined with 145 virgin females, was derived from offspring from the 250 mated founding females. This size of the control population produced parity with the experimental line in the number of genomic haplotypes transmitted across each generation. Other aspects of the culturing procedure of the control (such as density, days and times when flies were cultured) were matched with the experimental line.

FIG. 2 Fitness measures of experimental males exceed those of controls after 30 generations of evolution ($P_{dir} < 0.036$ for all comparisons, Student's *t*-tests, d.f. = 2, subscript 'dir' implies a directed test¹⁷ where 80% of the rejection region is in the anticipated direction as opposed to 100% or 50% as occurs in a 1- or 2-tailed test, respectively). All measures are standardized so that the average of the 2 control values is 1. Experimental populations are depicted by filled and control by open symbols; replicate A by squares and B by circles. Net fitness is the number of adult sons produced per adult male. Remating is the rate at which males mate with previously mated females. Defence is the extent to which a male's sperm continues to fertilize his mate's offspring after she is given the opportunity to remate with a competitor male. This measure increases through reduced remating by the male's mate and/or reduced displacement of his sperm when she remates.

METHODS. Net fitness. Extra vials were constructed by mixing EA, EB, CA' and CB' males with target females (4 and 8 vials each in generations 31 and 32, respectively) with a procedure matching that of the adapting-male compartment of Fig. 1 except that 75% of the males were replaced by competitor males that were homozygous for the marker *p^p* (previously recombined into the genetic background of the LH source stock). Offspring from competitor males were pink eyed and could be distinguished from red-eyed males carrying the genomic haplotype of the experimental (EA or EB) or control (CA' or CB') males. The ratio of red-eyed to pink-eyed adult male offspring, after adjusting for mendelian segregation, measured net relative fitness. **Remating.** In generation 34, observations were made of the mating behaviour of the flies in the adapting-male compartment of Fig. 1. There were 6 vials (2.5 cm × 17.5 cm tall, containing 43 males and 32 virgin target females) each for CA' and CB', and 18 vials each for EA and EB. Within 25 min of mixing the sexes, virtually every female was observed to be copulating. Four hours after combining the sexes, each vial of flies was observed (without handling the vials) and the number of copulating pairs per vial (rarely in excess of 4) was recorded; 16 such observations were made, each separated by at least 20 min, and spanning the first two days and



nights. Night observations were made with the aid of a red head lamp. **Defence.** In generation 36, 140 virgin target females were mixed with 168 experimental or control males (EA, EB, CA' or CB'; 7 vials each of 20 females and 24 males) and then the males were removed after 2 h by anaesthetizing the flies with CO₂ for 60 s. Virtually every female mated, with minimal opportunity for remating. Three hours after the first males were removed, *p^p* marked competitor males (see above) were added (24 per vial) to replace the original males. Males and females were mass transferred to fresh vials once without CO₂ anaesthesia after 24 h and then removed 24 h later. Progeny produced from the two sets of vials were scored for phenotype. The ratio of daughters with the target female phenotype to total *p^p* offspring represents a combined measure, which is not confounded by other components of male fitness, of a male's ability to inhibit female remating, as well as his ability to resist sperm displacement in those females that remated.

increased capacity for experimental males to remate with females previously mated by competitors (remating), and decreased capacity for competitor males to remate with females previously mated by experimental males, and displace extant sperm (defence). The enhanced defence capacity of the experimental males is probably attributable to a change, either qualitative or quantitative, in the

seminal fluid, as experimental males were not present when females which they had inseminated (during a brief 2-h period) were later challenged with competitor males.

The observed increase in adaptation of experimental males is associated with a substantial decrease in the survival of their mates, suggesting sexually antagonistic adaptation (Fig. 3). The

impact of EB males was larger than that of EA males. These results led me to question what was responsible for the negative impact on females, and why the experimental line EB harmed females more than EA.

Experimental EA and EB males did not induce higher fecundity in their mates compared with controls ($P_{\text{dir}} = 0.615$, Student's t -test, d.f. = 2), so elevated female fecundity cannot explain the toxic effect of experimental males. Alternatively, a higher remating rate of EA and EB males could lower female survival owing to the previously demonstrated toxic effects of seminal fluid^{4,5}. To test this, mortality and remating behaviour were measured simultaneously in generation 34 (Fig. 4). The experimental males (EA and EB) remated target females significantly more frequently than did controls (CA' and CB'; Fig. 2), but EB males did not remate females significantly more than did EA males ($P_{\text{dir}} = 0.33$, paired Student's t -test, d.f. = 15). There was a significant correlation between female death rate and remating rate (Fig. 4, dashed line; $P_{\text{dir}} = 0.0258$, Pearson's correlation, d.f. = 2), and there was marginally significant evidence that experimental line EB caused higher female mortality than predicted by remating rate in the other three lines (solid line; $P_{\text{dir}} = 0.068$, analysis of covariance (ANCOVA), d.f. = 1), possibly because of increased toxicity of their seminal fluid.

To confirm this marginally significant result, 7 samples of 20 virgin females each were mixed with 24 males each from EA, EB, CA', or CB' (data taken during the defence assay of Fig. 2). After only 2 hours the males were removed and replaced with males from an unrelated stock (the p^p competitor stock; see Fig. 3). As predicted from the earlier ANCOVA, the mortality of target females was substantially higher after short-term exposure to experimental EB males (mortality rate 13.3%) compared with the three other lines (mortality rate 5.6, 6.7 and 7.8% in lines CA', CB' and EA, respectively; $P_{\text{dir}} = 0.022$, F -test, d.f. = 1, 2).

In summary, experimental males evolved increased net fitness in response to the fixed phenotype of target females. Adaptation by experimental males resulted in a marked decrease in the survival of their mates with no compensating increase in female fecundity. The decreased female survival is associated, in both experimental lines, with an elevated rate of remating, but EB males further reduce female survival by inducing higher mortality per insemination.

The observed rapid and substantial responses of males to a static female phenotype demonstrates that sexually antagonistic

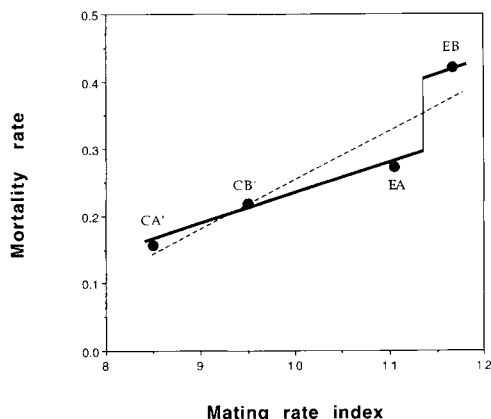


FIG. 4 Female death rate is positively associated with remating rate (dotted line) and the mortality induced by EB males exceeds the prediction based on remating in lines CA', CB', and EA (solid line).

METHODS. In generation 34, both mortality and remating rate were simultaneously measured in vials ($N = 18$ each for EA and EB and $N = 6$ each for CA' and CB') containing 32 target females and 43 males (see Fig. 2 legend). Mortality rate is the average fraction of females dying per vial, during the first two days after the males and virgin target females were combined. Remating index is the average number of rematings observed per vial, as described in Fig. 2.

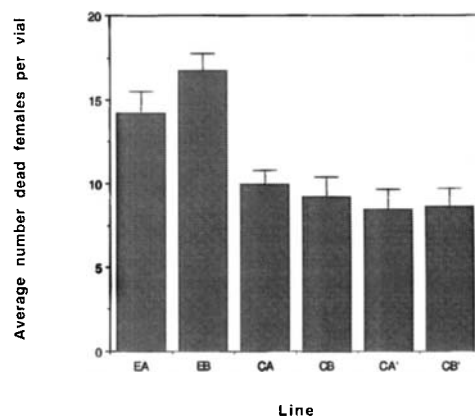


FIG. 3 The mortality of target females is increased when exposed to experimental compared with control males ($P_{\text{dir}} = 0.0194$, 0.028, respectively for experimental versus CA and CB or CA' and CB' controls, directed Student's t -tests, d.f. = 2).

METHODS. In generation 41, target females were combined, as usual, with males from the adapting-male line and mortality was measured for 2 days, the period when females produced offspring to be used in the next generation. In parallel, this same procedure was carried out using males from the controls both with (CA' and CB') and without (CA and CB) the translocation genetic background of the experimental males. The observed higher female mortality induced by experimental EB compared with EA males in generation 41 was also observed consistently in seven previous, longer-term (3–6 day) mortality assays in generations 29–32, 34, 36 and 37 ($P_{\text{dir}} = 0.0049$, directed binomial test), as was the higher mortality of both experimental lines compared with the two controls ($P_{\text{dir}} = 0.0003$, directed binomial test, data for controls was not measured in generation 36).

selection can be strong. The significance and extent of antagonistic coevolution between the sexes may be unappreciated as evidence for its previous operation is obscured by evolutionary *rapprochement*. Evidence for conflict between the sexes^{4,5} may therefore portend a history of chronic and substantial intersexual coevolution. Because each sex is a counteracting 'moving target', their coevolution may parallel that between a species and its parasites, predators and competitors¹¹, with the constraint that alleles that are too strongly antagonistic cannot evolve unless they are sex-limited in expression. Intersexual coevolution, therefore, would be far more extensive than adaptation to the physical environment, and contribute substantially to genetic divergence among physically isolated or semi-isolated populations^{12–15}. Many of the changes will affect reproductive physiology and behaviour, so that sexually antagonistic coevolution may be an important 'engine of speciation'¹⁶. □

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- Clarke, A. G., Aguade, M., Prout, T., Harshman, L. G. & Langley, C. H. *Genetics* **139**, 189–201 (1995).
- Harshman, L. G. & Prout, T. *Evolution* **48**, 758–766 (1994).
- Chen, P. et al. *Cell* **54**, 291–298 (1988).
- Fowler, K. & Partridge, L. *Nature* **338**, 760–761 (1989).
- Chapman, T., Liddle, L. F., Kalb, M. J., Wolfner, M. F. & Partridge, L. *Nature* **373**, 241–244 (1995).
- Charlesworth, B. *Genet. Res.* **63**, 213–227 (1994).
- Charlesworth, D., Morgan, M. T. & Charlesworth, B. *Genet. Res.* **61**, 1289–1303 (1993).
- Barton, N. *Genetics* **140**, 821–841 (1995).
- Manning, J. T. & Thompson, D. J. *Acta biotheoret.* **33**, 219–225 (1984).
- Peck, J. R. *Genetics* **137**, 597–606 (1994).
- Van Valen, L. *Evol. Theory* **1**, 1–30 (1973).
- Coyne, J. *Nature* **355**, 511–515 (1992).
- Wu, C.-I. & Palopoli, M. F. A. *Rev. Genet.* **27**, 283–308 (1994).
- Orr, H. A. *Genetics* **139**, 1805–1813 (1995).
- Muller, H. J. *Biol. Symp.* **6**, 71–125 (1942).
- Rice, W. R. & Hostert, E. H. *Evolution* **47**, 1637–1653 (1993).
- Rice, W. R. & Gaines, S. D. *Trends Ecol. Evol.* **9**, 235–237 (1994).

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Transcription factor AP-2 essential for cranial closure and craniofacial development

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DURING closure of the neural tube in the mouse, transcription factor AP-2 (refs 1–4) is expressed in ectoderm and in neural-crest cells migrating from the cranial neural folds⁵. Cranial neural crest cells provide patterning information for craniofacial morphogenesis, generate most of the skull bones, and, together with placodal ectoderm, form the cranial ganglia^{6–8}. To study the role of AP-2 during embryogenesis, we undertook a targeted mutagenesis of the AP-2 gene in the mouse. Here we report that AP-2^{-/-} mice died perinatally with cranio-abdominoschisis and severe dysmorphogenesis of the face, skull, sensory organs and cranial ganglia. Failure of cranial closure between 9 and 9.5 days postcoitum coincided with increased apoptosis in the midbrain, anterior hindbrain and proximal mesenchyme of the first branchial arch, but did not involve loss of expression of *twist* or *Pax-3*, two other regulatory genes known to be required for cranial closure.

A genomic segment containing exon 5 of the AP-2 gene, which is common to all known murine AP-2 isoforms⁹, was replaced with a PGK-*neo* gene cassette by homologous recombination in murine embryonic stem (ES) cells (Fig. 1a). Blastocyst injections of AP-2^{+/-} ES cells produced germline-transmitting chimaeric mice from which healthy, fertile AP-2^{+/-} progeny were generated. AP-2^{-/-} mutants were not observed among weaned pups from heterozygous intercrosses (Fig. 1b), but were present at the normal mendelian ratio throughout embryogenesis (Fig. 1c, d). AP-2^{-/-} mutants died perinatally manifesting fully penetrant phenotypic defects that included cranio-abdominoschisis, midline facial clefting, a contorted, small axial skeleton, and severe dysmorphogenesis of the skull, eyes, ears and cranial ganglia.

In AP-2^{-/-} embryos, the cranial portion of the neural tube failed to close and the two hemispheres of the brain developed open with germinal layer facing outward (cranioschisis) (Figs 1d and 2). Expansion of the everted forebrain between 9.5 and 11.5 days postcoitum (d.p.c.) led to progressive lateral displacement of the facial primordia, as visualized by *in situ* hybridization with antisense probe to *Msx-1* (ref. 10), a homeobox gene expressed in distal parts of the facial prominences (Fig. 2a, b). Subsequent failure of medial nasal and mandibular prominences to undergo normal midline fusions resulted in full midline facial clefting (Fig. 2g–i). The optic cup was also apparently displaced with dysmorphic neural and pigmented retinal layers of the eye developing medially (Fig. 2h). The occasional presence of a lens rudiment (not shown) on the distal side of the ectopic retina suggested that lens induction was aborted owing to loss of contact between optic cup and head ectoderm during abnormal brain growth. Meckel's and hyoid cartilage primordia were evident (Fig. 2i, j). The cranial ganglia, especially the trigeminal ganglia (Fig. 2g), were severely hypoplastic; dorsal root ganglia (trunk neural-crest cell derivatives) were less affected (Fig. 2j).

Skeletal preparations of embryos at 16.5 d.p.c. indicated multi-

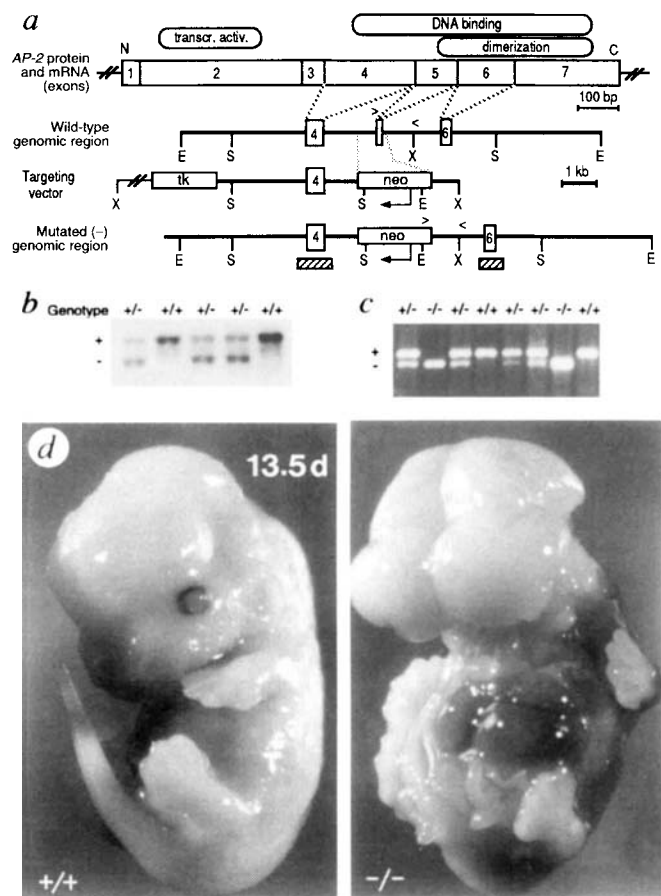


FIG. 1 Targeted disruption of the AP-2 gene in mice. **a**, AP-2 mRNA showing exonic and encoded protein functional domains. Scale bar, 100 base pairs. Second line, wild-type (+) genomic *EcoRI* (E) fragment containing AP-2 exons 4–6 (open boxes). The segment substituted with a *neo* gene in the targeting vector (shown linearized on third line) is indicated. S, *SacI*; X, *XhoI*. Bottom, ~850 bp of AP-2 chromosomal DNA including exon 5 is replaced with the *neo* gene by homologous recombination in ES cells. Scale bar, 1 kb. Hatched boxes designate Southern blot probes. **b**, Pups from F₁ +/– intercrosses were genotyped at weaning by tail DNA Southern blotting. No homozygous mutants (–/–) were observed among > 500 pups; heterozygous (+/–) and wild-type (+/+) mice were obtained at a ratio of ~2 : 1. **c**, AP-2^{-/-} homozygotes were present at expected frequencies throughout embryogenesis, as shown here by polymerase chain reaction (PCR) analysis of 9.5-d.p.c. embryos. **d**, Wild-type (left) and AP-2^{-/-} 13.5-d.p.c. embryos showing cranio-abdominoschisis, midline facial clefting, and absence of external eyes and ears.

METHODS. Murine AP-2 genomic clones were isolated from a strain 129/Sv genomic cosmid library. The targeting vector contains HSV-*tk* and PGK-*neo* cassettes^{24,25} flanked by AP-2 genomic DNA (5' arm, 5.1-kb *EcoRI*–*Bgl*III fragment containing exon 4; 3' arm, 0.9-kb *Bgl*III–*XhoI* intron 5 fragment). *XhoI*-linearized vector was electroporated into J1 ES cells. Homologous recombinants selected with G418 and FIAU were screened by Southern blotting (not shown). Germline-transmitting chimaeric males obtained from two +/– ES lines were bred to BALB/c and 129/Sv wild-type females to generate heterozygotes for intercrossing. Embryos were genotyped by yolk-sac DNA PCR²⁶; primers (denoted in a): 5'-TAATGTTGCTGGGTTGAAT-3' (<, common 3' primer); 5'-AACCAGAGCGAAGTCTAAGAA-3' (>, 5' primer, wild-type allele); 5'-ATTTGGGTTTGATATGCGTG-3' (>, 5' primer, mutated allele). Products: wild-type (+), 1.25 kb; mutated (–) allele, 1.1 kb.

ple bone and cartilage defects in AP-2^{-/-} mutants (Fig. 3). The head skeleton was acranic, and all other major skull bones of non-somitic origin⁷ were dysmorphic or reduced to remnants (Fig. 3b). Failure of the ventral body wall to close medially allowed extrusion of thoracic and abdominal organs through the open rib cage (Fig. 3d). Various limb defects were observed in AP-2^{-/-} mutants

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FIG. 2 Cranial closure defect and abnormal facial development in 11.5- and 13.5-d.p.c. $AP-2^{-/-}$ embryos. Side (a) and front (b) views of heads of 11.5-d.p.c. wild-type (left) and $-/-$ (right) embryos hybridized with *Msx-1* antisense probe. *Msx-1* is expressed in distal portions of facial prominences (n, medial and lateral nasal; x, maxillary; m, mandibular). In $-/-$ embryos, proximal portions of the prominences were greatly reduced (a) and distal portions were displaced laterally (b). c–j, Transverse head sections of 13.5-d.p.c. wild-type (c–f) and $AP-2^{-/-}$ (g–j) embryos (related wild-type region is shown above each $-/-$ section). In $-/-$ embryos, the forebrain (fb) extended over the clefted face, dysmorphic retinal (r) and pigmented-layer eye elements were located medially, the trigeminal ganglia (gV) and inner ear primordia (c, cochlear canal) were hypoplastic, the endolymphatic diverticulum (not shown) communicated directly to the exterior, and fourth ventricle choroid plexus (ch) was exposed externally between ectoderm and everted dorsal hindbrain neuroepithelium. Meckel's cartilage primordia (M) were apparent but the mandibular prominences (mn) remained unfused medially; hyoid cartilage primordia (h), tooth primordia (tp), and follicles of vibrissae (not shown) were present. Ear pinnae were not evident; middle-ear cartilage primordia were underdeveloped; ep, ependymal (germinal) brain layer; fn, frontonasal-derived; my, myelencephalon; p, anterior pituitary primordia; gVIII, vestibulocochlear ganglion; n, nasal cavity epithelia; a, atlas; b, first branchial cleft; mx, maxillary portion of first branchial arch; sc, spinal cord; t, tongue; drg, dorsal root ganglia; ph, pharynx; pmx, maxillary palatal shelf.

METHODS. Embryos from staged $AP-2^{-/-}$ intercrosses were collected and processed for whole-mount *in situ* hybridization²⁷ with digoxigenin (DIG)-labelled *Msx-1* RNA probe or for paraffin sectioning and haematoxylin–eosin histology²⁸.

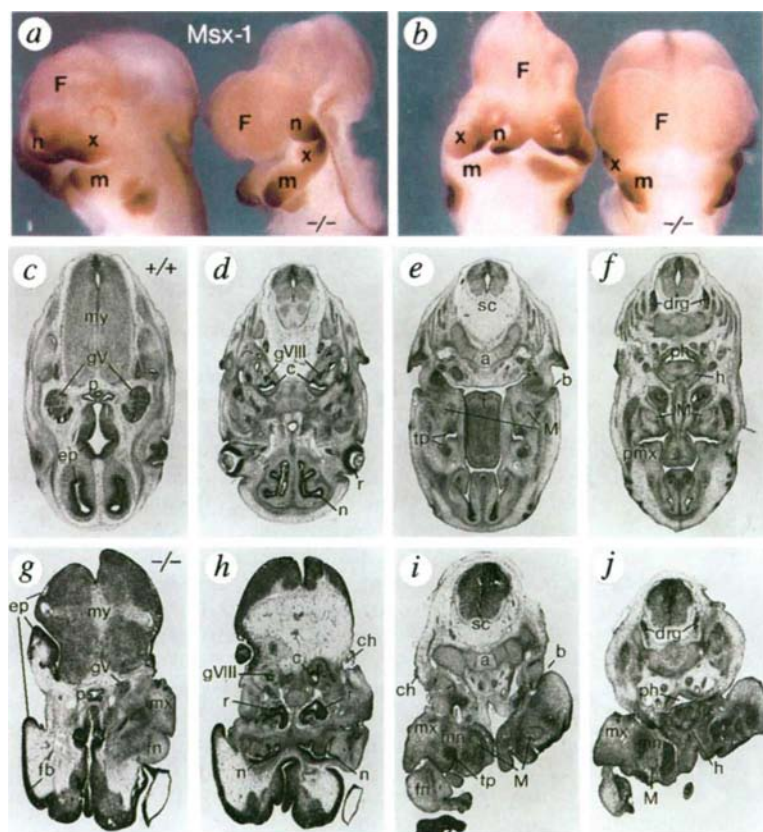


FIG. 3 Skeletal preps of 16.5-d.p.c. embryos. a, b, Sagittal views of wild-type and $AP-2^{-/-}$ head skeletons. c, d, Dorsal views of wild-type and mutant whole embryo skeletons. $AP-2^{-/-}$ mutants (b, d) were acranic (lack of skull vault, a consequence of exencephaly) and the remaining craniofacial skeleton was severely reduced and malformed. The maxilla and mandible were represented by a single, element on each side of the head with Meckel's cartilage on its ventromedial aspect. The axial skeleton was contorted and reduced in size, with scoliotic spine and no sternum medially. The ribs splayed outwards, but were unchanged in number. The arrowhead in d indicates forelimb lacking a radius bone. Somitic bones: At, atlas; Ax, axis; Ex, exoccipital. Major neural crest mesodermal derivatives of the first branchial arch: Ma, mandible; Mc, Meckel's cartilage; Mx, maxilla.

METHODS. Skeletons were prepared and stained with alizarin red (ossified bone) and alcian blue (cartilage) as described²⁸.

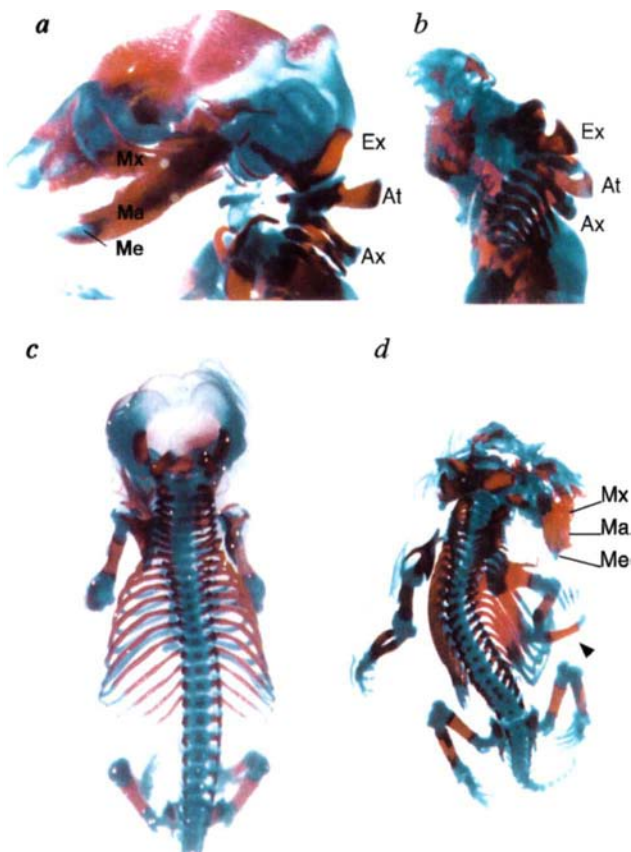
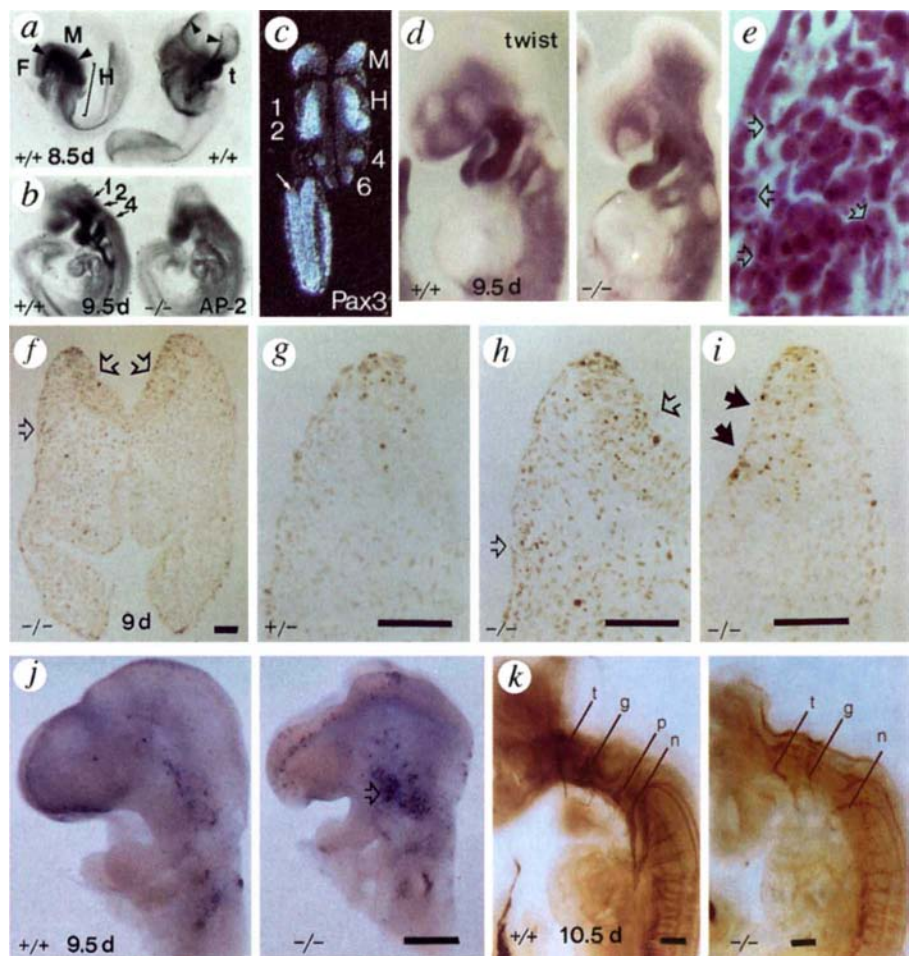


FIG. 4 Analysis of embryos at 9–9.5 d.p.c. a, b, Wild-type 8.25- (side view, left) and 8.5-d.p.c. (dorsal view, right) embryos hybridized with DIG-labelled *AP-2* antisense probe showing *AP-2* expression in cranial folds (arrowheads), emigrating cranial neural crest cells, and ectoderm. F, forebrain; M, mid-brain; H, hindbrain (bracket); t, trigeminal neural crest cells (first branchial arch). b, In 9.5-d.p.c. wild-type embryos (left), *AP-2* is expressed in neural crest cells emigrating from rhombomeres 1–2 (trigeminal neural crest) and 4 (facioacoustic neural crest) into branchial arches 1 and 2, respectively. In 9.5-d.p.c. *AP-2*^{-/-} embryos (right), greatly reduced *AP-2* expression correlated with failed cranial closure. c, *In situ* hybridization with ³⁵S-labelled *Pax-3* antisense probe to frontal sections of 9.5-d.p.c. *AP-2*^{-/-} and wild-type embryos (latter not shown) revealed essentially similar patterns of *Pax-3* expression in the dorsal brain plate, emigrating neural crest cells (M, mid-brain; H, hindbrain with neural crest-generating rhombomeres numbered) and in the posterior neural tube (arrow). Owing to breakage during embedding, the posterior part of the embryo is shifted left. d, Whole-mount *in situ* hybridization with DIG-labelled *twist* antisense probe indicated that *twist* RNA was still present in 9.5-d.p.c. *AP-2*^{-/-} embryo head mesenchyme (right). e, Haematoxylin–eosin-stained section from a 9-d.p.c. *AP-2*^{-/-} embryo showing pyknotic, fragmented nuclei indicative of apoptosis in proximal first branchial arch mesenchyme. f–i, TUNEL analysis on sections of early 9-d.p.c. wild-type (+/-, g) and -/- (f, h, i) embryos with 12–13 somites identified increased apoptosis in -/- embryos in the proximal part of the first branchial arch (small arrow), as well as in nearby neuroepithelial cells of the anterior hindbrain (larger arrow) and midbrain (filled arrows in i). Transverse sections in f–h pass through the anterior rhombencephalon neural folds and emerging trigeminal neural crest, optic vesicles and forebrain (lower neural folds). Scale bar in f–i, 50 µm. j, Whole-mount TUNEL of 9.5-d.p.c. wild-type (left) and -/- (right) embryos revealed increased cell death (arrow) in the trigeminal ganglion primordium and proximal first branchial arch in -/- mutants. k, Cranial ganglia deficiencies detected by immunostaining of 10.5-d.p.c. wild-type (left) and -/- mutant



(right) embryos with anti-neurofilament antibody. The petrosal ganglion (p) was missing, and the trigeminal (t), geniculate (g) and nodose (n) ganglia were severely reduced. Scale bar in j, k, 300 µm. METHODS. Embryos for analyses were genotyped by yolk-sac PCR²⁶ and matched by somite number. Haematoxylin–eosin histology²⁸, TUNEL analysis¹⁵, and *in situ* hybridization²⁷ with paraffin sections were performed as described, except that Vectastain ABC reagent (Vector Laboratories, Burlingame, CA) and DAB substrate were used for the TUNEL. Whole-mount *in situ* hybridization²⁷, TUNEL²⁹ and neurofilament immunostaining³⁰ were performed as described.

(Fig. 3d), although they were not fully penetrant on the current genetic backgrounds.

The earliest morphological abnormalities in *AP-2*^{-/-} embryos were evident by late 8.5 to early 9 d.p.c. and included delayed elevation of the cranial neural fold and a more widely open brain plate than were found in wild-type embryos of equivalent somite number (not shown). In wild-type embryos, *AP-2* is strongly expressed in the converging cranial folds and emigrating cranial neural crest cells (Fig. 4a, b). In *AP-2*^{-/-} mutants, the cranial folds failed to join dorsomedially for closure between 9 and 9.5 d.p.c., and the entire brain plate remained open thereafter.

To determine whether the defect in cranial closure was caused by loss of expression of *Pax-3* (ref. 11) or *twist* (ref. 12), two other transcription factors that are expressed in cranial neural crest cells and known to be required for cranial closure, the expression patterns of these genes were analysed by *in situ* hybridization. The results showed that some neural crest cells were still able to

migrate from the brain plate into the facial prominences in *AP-2*^{-/-} embryos at 9.5 d.p.c., and that expression of *Pax-3* (Fig. 4c) and *twist* (Fig. 4d) in these cells was not dependent on *AP-2*. Because expression of *AP-2* is also retained in the cranial neural crest cells of *twist*^{-/-} embryos at 9.5 d.p.c. (ref. 12), *twist* and *AP-2* do not appear to regulate each other, although they could potentially cooperate to activate other gene(s) required for cranial closure. Whether the partly penetrant hindbrain-closure defect in *Pax-3*-mutant (Spotch) mice¹¹ is caused by partial loss of *AP-2* expression remains to be examined.

Haematoxylin–eosin histology and TUNEL (terminal transferase-mediated dUTP nick end-labelling) analysis¹³ revealed two sites of increased apoptosis in head regions of *AP-2*^{-/-} embryos at 9 d.p.c. that correlated with onset of the cranial-closure defect. One site corresponded to mesenchymal cells in the vicinity of the future trigeminal ganglion primordium (Fig. 4e, f, h), and the second involved neuroepithelial cells scattered throughout the dorsal half of the anterior hindbrain (Fig. 4f, h) and midbrain (Fig. 4i). At 9.5

d.p.c., cell death was still elevated in the trigeminal ganglia primordia and proximal first-branchial-arch mesenchyme, but not in brain neuroepithelia (Fig. 4j). Immunostaining of embryos at 10.5 d.p.c. with anti-neurofilament antibody to label axons of peripheral-nervous-system neurons indicated that cranial ganglia were significantly underdeveloped (trigeminal, geniculate, vestibulocochlear and nodose) or missing (petrosal) in *AP-2*^{-/-} mutants (Fig. 4k).

The increased cell death in brain neuroepithelia at (or before) 9 d.p.c. could potentially be the direct cause of failed cranial closure, in that loss of neuroepithelial continuity may hinder elevation and apposition of the cranial neural folds¹⁴. The latter cell death is non-cell autonomous as *AP-2* is not expressed in these cells at this time. In contrast, the apoptosis in the trigeminal ganglia primordia and proximal mesenchyme of the first branchial arch may reflect cell-autonomous requirements for *AP-2*. Because *AP-2* is normally expressed in ectoderm and cranial neural crest cells, defective extracellular signalling from either of these cell types could potentially underlie the brain-plate apoptosis. The dimorphogenesis of neural-crest-derived skull bones may be multifactorial, with some aspects being caused by cell-autonomous deficiencies in neural-crest-derived mesectoderm or head ectoderm, and others (such as lack of skull vault) being secondary consequences of exencephaly.

The genetics of craniofacial morphogenesis have considerable developmental, evolutionary and medical implications¹⁵⁻¹⁷. As well as the targeted mutation of *AP-2* presented here, other gene-disruption experiments in mice have revealed roles in craniofacial patterning for transcriptional factors that include the retinoic acid receptors α and γ ¹⁸, the HLH-domain proteins Twist¹² and HES-1 (ref. 19), and homeodomain proteins Dlx-2 (ref. 20), Msx-1 (ref. 11), MHOx (ref. 21) and Gsc^{22,23}. These and related analyses are beginning to define the gene-expression programs that regulate craniofacial development. □

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- Mitchell, P. J., Wang, C. & Tjian, R. *Cell* **50**, 847–861 (1987).
- Williams, T., Admon, A., Lüscher, B. & Tjian, R. *Genes Dev.* **2**, 1557–1569 (1988).
- Lüscher, B., Mitchell, P. J., Williams, T. & Tjian, R. *Genes Dev.* **3**, 1507–1517 (1989).
- Williams, T. & Tjian, R. *Genes Dev.* **5**, 670–682 (1991).
- Mitchell, P. J., Timmons, P. M., Hébert, J. M., Rigby, P. W. J. & Tjian, R. *Genes Dev.* **5**, 105–119 (1991).
- Le Douarin, N. *The Neural Crest* (Cambridge Univ. Press, 1982).
- Couly, G. F., Coltey, P. M. & Le Douarin, N. *Development* **117**, 409–429 (1993).
- D'Amico, M. A. & Noden, D. M. *Am. J. Anat.* **166**, 445–468 (1983).
- Meier, P., Koedood, M., Philipp, J., Fontana, A. & Mitchell, P. J. *Dev. Biol.* **169**, 1–14 (1995).
- Satokata, I. & Maas, R. *Nature Genet.* **6**, 348–356 (1994).
- Epstein, D. J., Vekemans, M. & Gros, P. *Cell* **67**, 767–774 (1991).
- Chen, Z.-F. & Behringer, R. R. *Genes Dev.* **9**, 686–699 (1995).
- Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. *J. Cell Biol.* **119**, 493–501 (1992).
- Gordon, R. J. *Embryol. exp. Morph.* **89**, (suppl.) 229–255 (1985).
- Richman, J. & Mitchell, P. J. *Curr. Biol.* **6**, 364–367 (1996).
- Gans, C. & Northcutt, R. G. *Science* **220**, 268–274 (1983).
- Copp, A. J. & Bernfield, M. *Curr. Opin. Pediatr.* **6**, 624–631 (1994).
- Lohnes, D. et al. *Development* **120**, 2723–2748 (1994).
- Qiu, M. et al. *Genes Dev.* **9**, 2523–2538 (1995).
- Ishibashi, M. et al. *Genes Dev.* **9**, 3136–3148 (1995).
- Martin, J. F., Bradley, A. & Olsen, E. N. *Genes Dev.* **9**, 1237–1249 (1995).
- Yamada, G. et al. *Development* **121**, 2917–2922 (1995).
- Rivera-Pérez, J. A., Mallo, M., Gendron-Magure, M., Gridley, T. & Behringer, R. R. *Development* **121**, 3005–3012 (1995).
- Mansour, S. L., Thomas, K. & Capocchi, M. *Nature* **336**, 348–352 (1988).
- Soriano, P., Montgomery, C., Geske, C. & Bradley, A. *Cell* **64**, 693–702 (1991).
- Hogan, B., Beddington, R., Costantini, F. & Lacy, E. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory Press, NY, 1994).
- Wilkinson, D. G. *In Situ Hybridization: a Practical Approach* (IRL, Oxford, 1992).
- Kaufman, M. H. *The Atlas of Mouse Development* (Academic, London, 1992).
- Conlon, R. A., Reaume, A. G. & Rossant, J. *Development* **121**, 1533–1545 (1995).
- Emfors, P., Lee, K. F. & Jaenisch, R. *Nature* **368**, 147–150 (1995).

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Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2

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THE retinoic acid-inducible transcription factor AP-2 is expressed in epithelial and neural crest cell lineages during murine development¹⁻⁵. AP-2 can regulate neural and epithelial gene transcription, and is associated with overexpression of c-erbB-2 in human breast-cancer cell lines⁴⁻⁶. To ascertain the importance of AP-2 for normal development, we have derived mice containing a homozygous disruption of the *AP-2* gene. These *AP-2*-null mice have multiple congenital defects and die at birth. In particular, the *AP-2* knockout mice exhibit anencephaly, craniofacial defects and thoraco-abdominoschisis. Skeletal defects occur in the head and trunk region, where many bones are deformed or absent. Analysis of these mice earlier in embryogenesis indicates a failure of cranial neural-tube closure and defects in cranial ganglia development. We have shown that AP-2 is a fundamental regulator of mammalian craniofacial development.

Mice containing a disrupted *AP-2* gene were generated by standard procedures (Fig. 1a, b). Most heterozygous mice appeared normal, apart from a higher incidence of dental malocclusions (4%). Homozygous *AP-2*-null mice died at or before birth as a result of severe congenital defects (Fig. 1). They had anencephaly and lacked ventral craniofacial structures, including a recognizable mouth, snout or ears; they also displayed thoraco-abdominoschisis, in which the ventral body wall was absent and abdominal and thoracic contents were exposed (Fig. 1c). However, there was no general failure of body wall formation as mature keratinized skin, containing hair follicles, covered the dorsal surface of the torso. Thus absence of AP-2 disrupted development and migration of the dorsolateral body wall that normally generates ventral surface⁷. Homozygous embryos were also runted compared with normal littermates, consistent with hypoplastic and retarded development of several tissues and organs, including heart and kidney (not shown). Analysis of multiple litters indicated complete penetrance of the homozygous phenotype.

Skeletons of *AP-2*-null mice exhibited thoracic abnormalities including scoliosis, missing clavicles, and a rib cage that was not fused to a central sternum (Fig. 2a, b). Tissues necessary for development of sternum and clavicles are absent owing to thoraco-abdominoschisis. Developing sternal rudiments originate in the dorsolateral body wall and migrate with this tissue towards the ventral midline, eventually fusing to form the sternum⁷. Clavicle formation requires an interaction between preclavicular mesenchyme and overlying epidermis⁸. Several mice also had forelimb defects; in particular, 18 of 28 knockout embryo forelimbs lacked a radius (Fig. 2b). This finding is consistent with expression of AP-2 in limb buds¹, and suggests it shares a common pathway with *Hox* genes in forelimb development⁹.

AP-2 knockout embryos displayed major alterations in craniofacial morphology (Fig. 2c–e). The cephalic skeleton can be classified as chordal, associated with the notochord, and achordal, rostral to this boundary¹⁰. Chordal skeleton is derived from

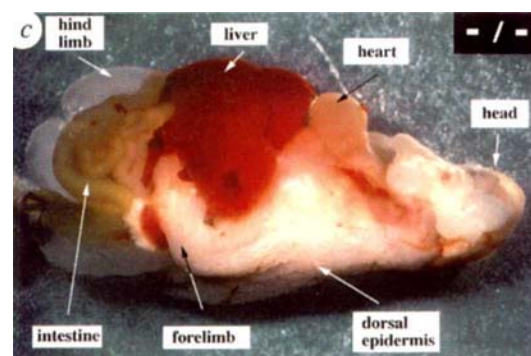
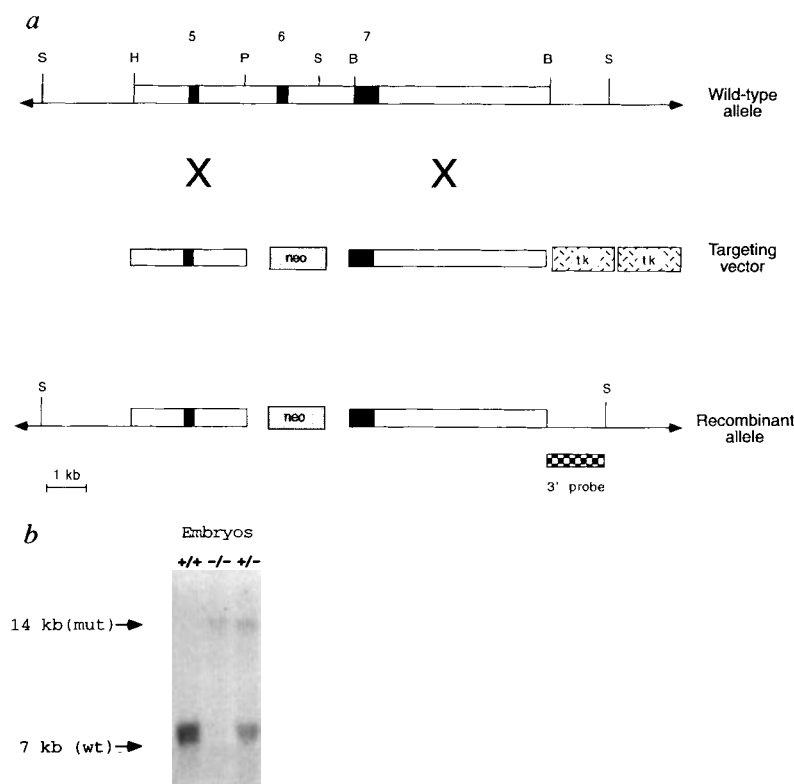


FIG. 1 Generation of mice deficient in AP-2. *a*, The wild-type AP-2 gene (top), targeting vector (middle), and recombinant locus (bottom). Exons 5, 6 and 7 (black boxes), restriction enzyme sites (S, *SacI*; H, *HindIII*; P, *PstI*; B, *BglII*), and the 3' probe are shown. *b*, Southern blot of *SacI*-digested DNA from wild-type (+/+), knockout (-/-) and heterozygous (+/-) embryos probed with a 3' probe. The 7-kb wild-type allele (wt) is converted to a 14-kb fragment by homologous recombination. The organization of the recombinant allele was also confirmed using a 5' probe (data not shown). *c*, *d*, Lateral view of the AP-2 knockout mouse (*c*; -/-) and a wild-type littermate (*d*; wt/wt) at day E19.5.

METHODS. An AP-2 clone was isolated from a 129/SV genomic library and used to construct the targeting vector. A 2.9-kb *HindIII*-*PstI* 5' fragment was inserted into pSP73 (Promega) adjacent to an *XhoI*-*BamHI* fragment from

pMC1neoPA (Stratagene) cloned between *Sall* and *BamHI*. A 5-kb *BglII* 3' AP-2 fragment was then inserted into the *BamHI* site next to a *Clal*-*BamHI* fragment containing two copies of the herpes simplex virus thymidine kinase gene. The resulting clone lacks exon 6 and the first 3 nucleotides of exon 7, destroying the dimerization domain of AP-2 that is critical for DNA binding^{25,26}. The construct was linearized with *Clal* before electroporation and drug selection²⁷. Embryonal stem-cell clones that had undergone homologous recombination were identified by Southern blotting and injected into C57BL/6/J blastocysts. Male chimaeric mice were bred to Black Swiss females (Taconic). Genotypes of subsequent offspring were determined by Southern blotting or polymerase chain reaction (PCR). RNase protection also indicated the presence of a transcript diagnostic for the disrupted AP-2 gene.

cephalic and somitic mesoderm; achordal skeleton is neural crest in origin^{10,11}. In knockout mice, the axis and atlas were normal. Exoccipital, basioccipital and basisphenoid bones of the chordal skeleton were also identifiable. However, a subset of bones of NCC origin were observed. The hyoid bone was indistinguishable from wild type, and structures resembling Meckel's cartilage and mandibles were observed, although the latter were unfused.

In the nervous system, dorsal root ganglia and several cranial ganglia contain a NCC component that expresses AP-2 (ref. 1), and these were analysed using an anti-neurofilament antibody (Fig. 2*f, g*). At embryonic day 10.5 (E10.5), dorsal root ganglia appeared normal in knockout embryos. In contrast, the trigeminal ganglion (V) was greatly reduced and the oculomotor nerve (III) was not seen. The morphologies of the VII/VIII ganglion complex, and IX and X ganglia and nerves, were also disrupted with variable penetrance. These phenotypes are also consistent with alterations in cranial NCC fate.

No gross anatomical differences could be identified between

knockout mice and littermates at E8.5 of gestation (not shown). At E9.5 of normal development, the neural folds are apposed and fused over most of the spinal and cranial region¹² (Fig. 3*a, c*). However, in knockout mice, cranial neural folds remained widely separated over their entire rostral-caudal axis, resulting in exencephaly (Fig. 3*b, d*). Closure of spinal neural tube appeared normal. The cranial neural tube defect was exacerbated during further development, as neuroepithelial tissue continued to proliferate. By E12.5, the knockout mouse head was largely neuroepithelium (Fig. 3, compare *e* and *f*).

AP-2 is normally concentrated in cranial NCC and their derivatives; homozygous mutation abolishes this expression (Fig. 4*a, b*). Craniofacial defects could arise if AP-2 inhibited NCC formation. Therefore, the distribution of neural crest and neuroepithelial cells was examined by using an antibody that recognizes neural cell-adhesion molecules (N-CAM). In normal embryos, neuroepithelial cells expressing this epitope were observed at the apex of the neural folds (Fig. 4*c*). In knockout mice, the cranial staining pattern was considerably altered; positive cells were not restricted to the apices of the folds, but were observed as a large population lining the defective neural tube (Fig. 4*d*). In spinal neural tube, the staining pattern was indistinguishable from wild type. The distribution of migratory NCC was assayed by using a transgene that drives *lacZ* expression in these cells¹³. Both wild-

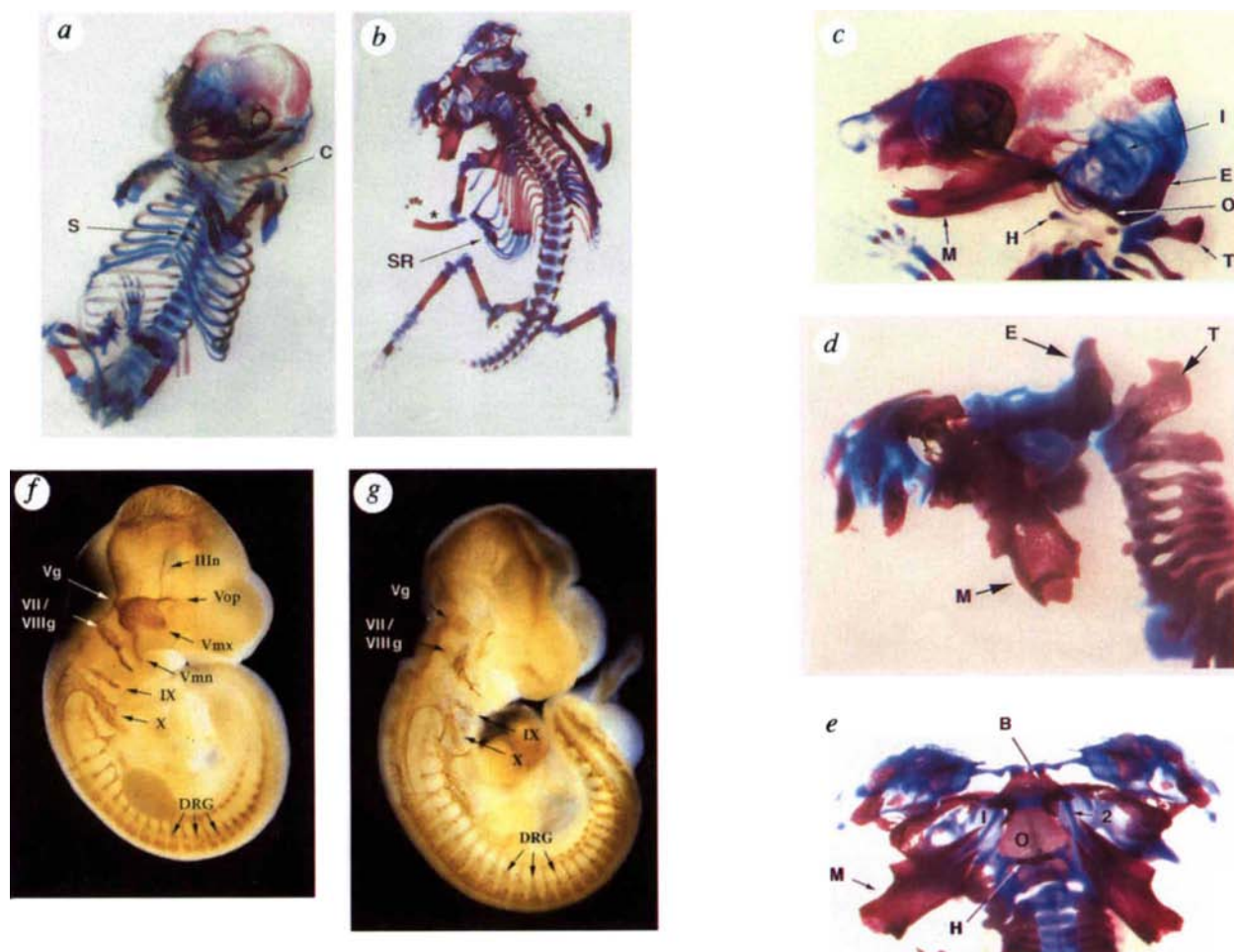


FIG. 2 Analysis of the AP-2 knockout mouse skeleton and nervous system. *a, b*, Ventral view of wild-type (*a*) and AP-2-null (*b*) embryos at E16.5. *c, d*, Lateral view of head of wild-type (*c*) and AP-2-null (*d*) embryos. *e*, Ventral view of head of AP-2-null embryo at E16.5. Meckel's cartilage (2), the cochlea (I), clavicle (C), atlas (T), exoccipital (E), basioccipital (O), basisphenoid (B), mandibles (M), sternum (S), sternal remnants (SR; disorganized bony tissue at the fused ends of the ribs on either side of the body that may represent a rudimentary split sternum), and hyoid (H) bones are indicated. The presence of only one bone in the zeugopod region of the

forelimb is indicated by an asterisk in the null embryo. *f, g*, Neurofilament staining of the wild-type (*f*) and AP-2-null (*g*) embryos. The cranial nerves, cranial ganglia and dorsal root ganglia (DRG) are indicated. Vop, Vmx and Vmn indicate the ophthalmic, maxillary and mandibular branches of the trigeminal nerve (=Vg) respectively.

METHODS. Staining with alcian blue (cartilage) and alizarin red (bone) performed essentially as described²⁸. Whole-mount immunostaining was as described²⁹.

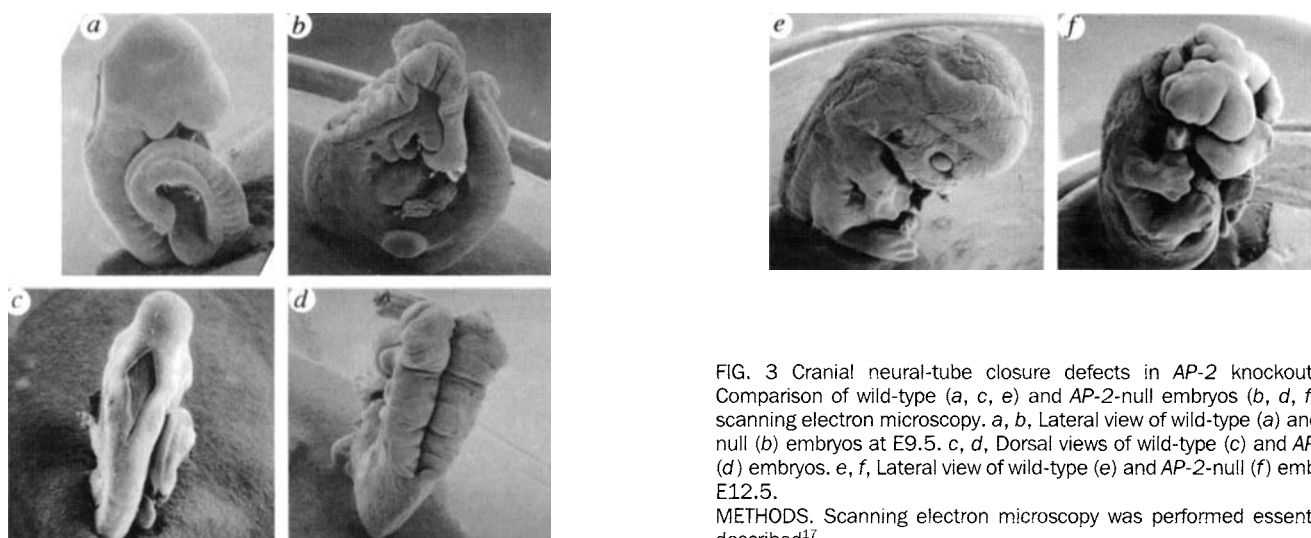


FIG. 3 Cranial neural-tube closure defects in AP-2 knockout mice. Comparison of wild-type (*a, c, e*) and AP-2-null embryos (*b, d, f*) using scanning electron microscopy. *a, b*, Lateral view of wild-type (*a*) and AP-2-null (*b*) embryos at E9.5. *c, d*, Dorsal views of wild-type (*c*) and AP-2-null (*d*) embryos. *e, f*, Lateral view of wild-type (*e*) and AP-2-null (*f*) embryos at E12.5.

METHODS. Scanning electron microscopy was performed essentially as described¹⁷.

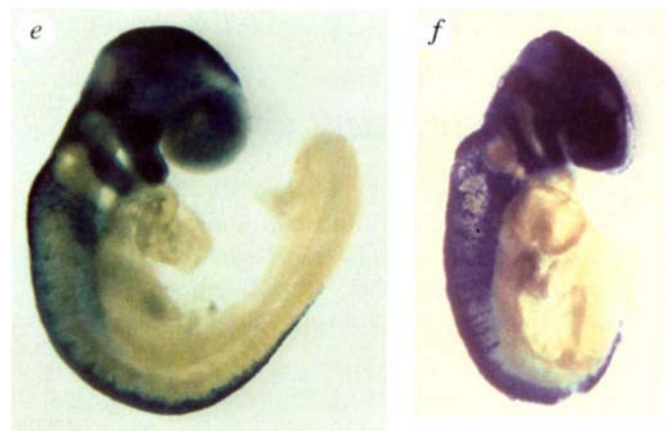


FIG. 4 Examination of presumptive neural crest in *AP-2* knockout mice. *a*, *b*, Lateral view of E9 wild-type (*a*) and E9.5 *AP-2*-null (*b*) embryos analysed using the *AP-2* monoclonal antibody. At this stage in the wild-type, *AP-2* is most apparent in the face, first and second branchial arches, and neural crest originating from rhombomeres 2 and 4 of the hindbrain. The staining in the tail is a secondary antibody-specific artefact. *c*, *d*, Dorsal view of E9.5 wild-type (*c*) and *AP-2*-null (*d*) embryos analysed using a monoclonal antibody specific for the HNK-1 epitope. *e*, *f*, Lateral view of E9.5 wild-type (*e*) and *AP-2*-null (*f*) embryos stained for expression of the *wnt-1-lacZ* fusion transgene in neural crest.

METHODS. Whole-mount immunohistochemistry was performed essentially as described³⁰, using an *AP-2* monoclonal antibody or the mouse monoclonal anti-HNK-1/N-CAM antibody, VC1.1 (Sigma), which recognizes N-CAM. The *AP-2* antibody recognizes an epitope N-terminal to the *neo* insertion and therefore indicates that no detectable peptide is produced by the disrupted gene. Whole-mount β -galactosidase staining of mice carrying the *Wnt-1-lacZ* reporter was performed as described¹³. Expression of this transgene occurs in NCC both before and after they migrate from the neural tube.

type and knockout embryos carrying this transgene display indistinguishable staining of migratory NCC (Fig. 4*e, f*). Taken together with the skeletal analysis, these data indicate that the NCC are migratory and can differentiate into a subset of craniofacial structures in knockout mice. Furthermore, this analysis demonstrates that the neural-tube closure defect does not result from inhibition of NCC migration^{14,15}. Instead, neural crest/neurepithelial cells must regulate neural-tube closure by an alternative mechanism, for example excess neurepithelial proliferation, which prevents correct neural-fold morphogenesis. Furthermore, craniofacial abnormality arises partly from neurepithelial overgrowth, for example eyes are present in knockout mice but are imbedded in neural tissue (not shown). However, alterations are also observed in a subset of NCC derivatives indicating that a

population of these cells fail to develop into their appropriate craniofacial structures at their normal destination. This failure of craniofacial development is accompanied by cell death associated with the trigeminal ganglia at E9.5, and with the face region at E12.5 (not shown).

Disruption of *AP-2* is one of the few mouse mutations that invariably cause neural-tube closure defects^{16–18}, which can be associated with ventral body wall abnormalities¹⁹. The *AP-2*-null phenotype is also reminiscent of those caused by teratogens^{20–22}, including retinoic acid. The *AP-2* gene resides on human chromosome 6 and mouse chromosome 13, near several mutations that affect craniofacial and NCC development^{23,24}. *AP-2* provides an important tool to analyse the genetic and environmental causes of congenital defects. □

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- Mitchell, P. J., Timmons, P. M., Hébert, J. M., Rigby, P. W. J. & Tjian, R. *Genes Dev.* **5**, 105–119 (1991).
- Meier, P., Koedood, M., Philipp, J., Fontana, A. & Mitchell, P. J. *Dev. Biol.* **169**, 1–14 (1995).
- Lüscher, B., Mitchell, P. J., Williams, T. & Tjian, R. *Genes Dev.* **3**(10), 1507–1517 (1989).
- Byrne, C., Tainsky, M. & Fuchs, E. *Development* **120**, 2369–2383 (1994).
- Williams, T., Admon, A., Lüscher, B. & Tjian, R. *Genes Dev.* **2**, 1557–1569 (1988).
- Bosher, J. M., Williams, T. & Hurst, H. C. *Proc. natn. Acad. Sci. U.S.A.* **92**, 744–747 (1995).
- Chen, J. M. *J. Anat.* **86**, 373–386 (1952).
- Hall, B. K. *J. Embryol. exp. Morph.* **93**, 133–152 (1986).
- Davis, A. P., Witte, D. P., Hsieh-Li, H. M., Potter, S. S. & Capocchi, M. R. *Nature* **375**, 791–795 (1995).
- Le Douarin, N. M., Ziller, C. & Couly, G. F. *Dev. Biol.* **159**, 24–49 (1993).
- Hall, B. K. & Hörstadius, S. *The Neural Crest* (Oxford Univ. Press, 1988).
- Copp, A. J., Brook, F. A., Estibeiro, P., Shum, A. S. W. & Cockcroft, D. L. *Prog. Neurol.* **35**, 363–403 (1990).
- Echelard, Y., Vassileva, G. & McMahon, A. P. *Development* **120**, 2213–2224 (1994).
- Morris-Kay, G. & Chen, W.-H. *Neural Tube Defects* (ed. Bock, G. & Marsh, J.) 51–56 (John Wiley, Chichester, UK, 1994).
- Bronner-Fraser, M. *FASEB J.* **8**, 699–706 (1994).
- Copp, A. J. *Neural Tube Defects* (ed. Bock, G. & Marsh, J.) 118–134 (John Wiley, Chichester, UK, 1994).
- Chen, Z.-F. & Behringer, R. R. *Genes Dev.* **9**, 686–699 (1995).
- Günther, T., Struwe, M., Aguzzi, A. & Schughart, K. *Development* **120**, 3119–3130 (1994).

- Stumpo, D. J., Bock, C. B., Tuttle, J. S. & Blackshear, P. J. *Proc. natn. Acad. Sci. U.S.A.* **92**, 944–948 (1995).
- Morris-Kay, G. *Bioessays* **15**, 9–15 (1993).
- Kotch, L. E. & Sulik, K. K. *Am. J. med. Genet.* **44**, 168–176 (1992).
- Wei, X. & Sulik, K. K. *Am. J. med. Genet.* **47**, 862–871 (1993).
- Davies, A. F. et al. *Hum. molec. Genet.* **4**, 121–128 (1995).
- Warren, G., Gordon, M., Siracusa, L. D., Buchberg, A. M. & Williams, T. *Genomics* **31**, 234–237 (1996).
- Williams, T. & Tjian, R. *Genes Dev.* **5**, 670–682 (1991).
- Williams, T. & Tjian, R. *Science* **251**, 1067–1071 (1991).
- Kuida, K. et al. *Science* **267**, 2000–2003 (1995).
- Martin, J. F., Bradley, A. & Olson, E. N. *Genes Dev.* **9**, 1237–1249 (1995).
- Matsuo, I., Kuratani, S., Kimura, C., Takeda, N. & Aizawa, S. *Genes Dev.* **9**, 2646–2658 (1995).
- Mark, M. et al. *Development* **119**, 319–338 (1993).

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A cortical neuropeptide with neuronal depressant and sleep-modulating properties

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ACETYLCHOLINE (ACh) plays a key role in the transitions between the different phases of sleep¹: Slow-wave sleep requires low ACh concentrations in the brain, whereas rapid-eye-movement (REM) sleep is associated with high levels of ACh. Also, these phases of sleep are differentially sensitive to a number of endogenous neuropeptides and cytokines, including somatostatin, which has been shown to increase REM sleep without significantly affecting other phases². Here we report the cloning and initial characterization of cortistatin, a neuropeptide that exhibits strong structural similarity to somatostatin, although it is the product of a different gene. Administration of cortistatin depresses neuronal electrical activity but, unlike somatostatin, induces low-frequency waves in the cerebral cortex and antagonizes the effects of acetylcholine on hippocampal and cortical measures of excitability. This suggests a mechanism for cortical synchronization related to sleep.

While characterizing region-specific brain messenger RNAs, we isolated a complementary DNA clone whose sequence suggested that it corresponded to an mRNA that encoded a new protein of 112 amino-acid residues which we called preprocortistatin. Alignment of preprocortistatin with the 116-residue sequence of preprosomatostatin (Fig. 1) revealed several points of structural similarity, although the only extended amino-acid identity was at their carboxy termini. Preprocortistatin starts with a 27-residue apparent secretion-signal sequence. Cleavage of the preprospecies to procortistatin would produce a protein that could be proteolytically matured further at either of two tandem basic amino-acid pairs to produce cortistatin-29 and cortistatin-14, analogous to the cleavage of preprosomatostatin at 28 and 14 residues³, or at both basic pairs to produce cortistatin-13 as well. Cortistatin-13 is unrelated to any known species, whereas cortistatin-14 shares 11 of 14 residues with somatostatin-14, including two cysteines that may cause the peptide to cyclize, and the FWKT sequence that is critical for somatostatin binding to its receptors⁴. Cortistatin-14 and somatostatin-14 are permuted by one amino acid (the alignment of cortistatin begins at residue 2 of somatostatin; cortistatin terminates with a lysine that extends one residue beyond the C-terminal cysteine of somatostatin) (Fig. 1). This difference and their cDNA sequences indicate clearly that they are the products of separate genes.

FIG. 1 Predicted amino-acid sequence of preprocortistatin. Preprocortistatin's deduced amino-acid sequence begins with a putative secretion signal peptide, whose predicted cleavage site is indicated by an arrow. The only region of extended similarity between cortistatin and somatostatin lies in the C terminus. Alignment of cortistatin-29 (CST) and somatostatin-28 (SST) amino-acid sequences shows that both statins share the critical residues for binding to the receptors and the cysteines that are likely to render the peptides cyclic. The basic amino acids that are putative processing sites are boxed. Sequence alignment was performed with the BESTFIT program (GCG group, University of Wisconsin).

Somatostatin	1	MLSCRLQCALAALICIVLAIGGVTGAPSDPRLRQFLQKSLAAANTGKQELAKYFLAELLSEP
Cortistatin	1	MGGCSTRGKRPSALSLLLLLLSGIAASALPLESGPTGQDSVQDATGRRRTGLLT
		↑
		SST-28
Somatostatin	61	NQTENDALEPEDLPQAAEQDEMRLERNSANSNPAMAPRRRAGCKNFFWKTFSTSC
		SST-14
Cortistatin	55	FLAWHWEASQSSSTAFEGGTPELSEKQERPLQOPPHRDKRPCKNEFWKTFSSCK
		CST-14
		CST-29

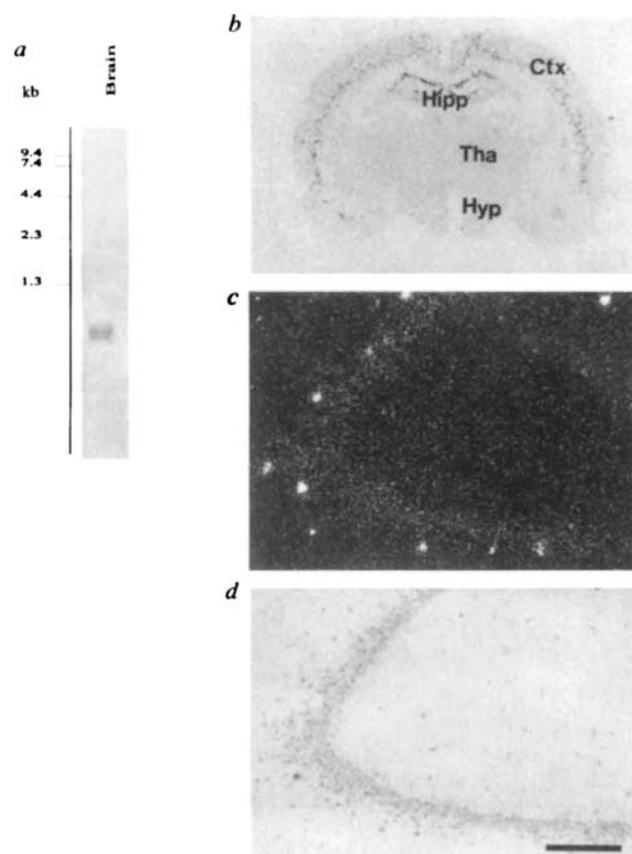


FIG. 2 Cortistatin is brain-specific. *a*, Northern blot containing 2 μ g poly(A)-selected RNA from rat brain. *b*, *In situ* hybridization autoradiograph showing a coronal section of a rat brain hybridized with a cortistatin riboprobe. Note that the signal is restricted to individual sparse cells in the cortex and hippocampus. No signals were detected in the hypothalamus, a physiologically important site of somatostatin expression. In the hippocampal formation, cortistatin expression was restricted to scattered non-pyramidal cells in the subiculum and stratum oriens of CA1 and CA3 (*c*; *d* is the brightfield image of *c*; scale bar, 200 μ m). *In situ* hybridization was done on free-floating sections as described¹⁸.

We determined the distribution of preprocortistatin mRNA by northern blotting. A single band of ~600 nucleotides was detected in samples prepared from brain (Fig. 2*a*), but not from adrenal gland, liver, spleen, thymus, ovary, testes or anterior pituitary (results not shown). We then examined the cellular distribution of cortistatin mRNA by *in situ* hybridization on rat brain sections. Signals were detected only in scattered cells throughout the cerebral cortex and hippocampus (Fig. 2*b*), but not in hypothalamus, an important site of somatostatin expression. In the hippocampus, we found cortistatin mRNA expression in some non-pyramidal cells in the subiculum and in the stratum oriens of the CA1 and CA3 fields, suggesting that cortistatin

mRNA might be present in GABAergic interneurons (Fig. 2c, d). We therefore performed double *in situ* hybridization experiments to detect co-expression of cortistatin and GAD₆₅/GAD₆₇ mRNAs, which encode the enzymes involved in GABA synthesis⁵. All cortistatin-expressing cells also contained either GAD₆₅ or GAD₆₇ mRNA, which was evidence for the GABAergic nature of these neurons (not shown).

We examined the binding of chemically synthesized, cortistatin-14 peptide to somatostatin receptors on GH₄ pituitary cells. Both cortistatin-14 and somatostatin-14 effectively displaced bound [¹²⁵I]-labelled somatostatin-14 in a dose-dependent manner with estimated K_d s, (5×10^{-9} M; Fig. 3a) very close to that previously reported for somatostatin⁶. To investigate whether cortistatin regulates activation of the somatostatin receptor, we determined the concentration of cyclic AMP following stimulation of GH₄ cells with vasoactive intestinal peptide (VIP) or thyroid-releasing hormone (TRH) in the presence or absence of either cortistatin or somatostatin. Both statin peptides effectively inhibited stimulation by VIP and TRH (Fig. 3c). Cortistatin or somatostatin alone had no effect on basal cAMP concentrations (Fig. 3b). Both peptides at 10^{-8} M completely inhibited TRH, but attenuated the effect of VIP in a dose-dependent manner by ~50% at 10^{-6} M. Cortistatin therefore appears to act as an agonist on the endogenous somatostatin receptors expressed by GH₄ cells, although these cells may express a heterogeneous population of receptors and the agonist activity may not necessarily be its function at its sites of expression.

We tested whether cortistatin had somatostatin-like effects on hippocampal neurons by using current- and voltage-clamp recordings in a hippocampal-slice preparation. Superfusion of cortistatin, like somatostatin-14, hyperpolarized these neurons (10 of 11 cells), in association with inhibition of action-potential firing (Fig. 4a), followed by recovery to control levels upon washout of the peptide. Unlike somatostatin, the cortistatin effect developed slowly, reaching a maximum 6–8 min after the onset of the response: somatostatin's effect on these neurons took 2–3 min to peak under the same conditions (not shown).

To determine the mechanism of this cortistatin-induced inhibition, we tested the effect of cortistatin on the M current (I_M), a non-inactivating voltage-dependent potassium current in hippocampal neurons⁷. Like somatostatin^{8,9}, cortistatin superfusion increased the amplitude of the I_M relaxation concomitantly with an outward steady-state current, with recovery to control levels upon washout (not shown).

The inhibition by cortistatin of excitability of CA1 pyramidal neurons was paralleled *in vivo* in the anaesthetized preparation. Field potentials were elicited in the CA1 region by stimulation of the commissural pathway. Stimulation of the monosynaptic afferent input to the CA1 region evoked a characteristic population spike (PS) that represents the synchronous firing of pyramidal cells, superimposed on a field synaptic potential waveform¹⁰. We generated stimulus–response curves to characterize the effects of cortistatin on extracellular recordings in CA1. Microiontophoretic application of cortistatin, like somatostatin, significantly decreased PS amplitudes both at half-maximal and maximal levels of stimulation (Fig. 4b).

As cortistatin is expressed in interneurons in the cerebral cortex and hippocampus, we investigated its effects on cortical measures of neuronal excitability. We infused up to 6 nmol HPLC-purified synthetic peptide into the brain ventricles of rats ($n = 5$) and recorded the electroencephalogram (EEG) for 4 hours after peptide injection. In addition, rats were observed for changes in spontaneous behaviour through a one-way window. Cortistatin-treated animals were clearly hypoactive compared with saline-injected rats, but kept their eyes open and displayed other signs of wakefulness for a short period (15–20 minutes). In these animals, the EEG showed a dramatic increase in cortical slow waves (1–4 Hz). Polygraphic monitoring of arousal states after cortistatin administration (Fig. 4c) also indicated that rats spent up to 75% of the 4-hour recording time in slow-wave sleep compared to 40%

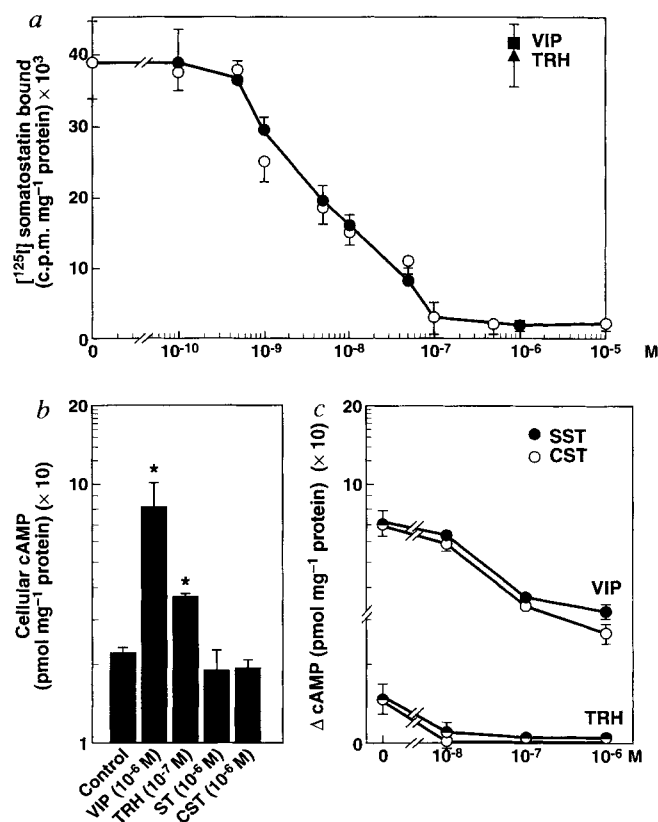


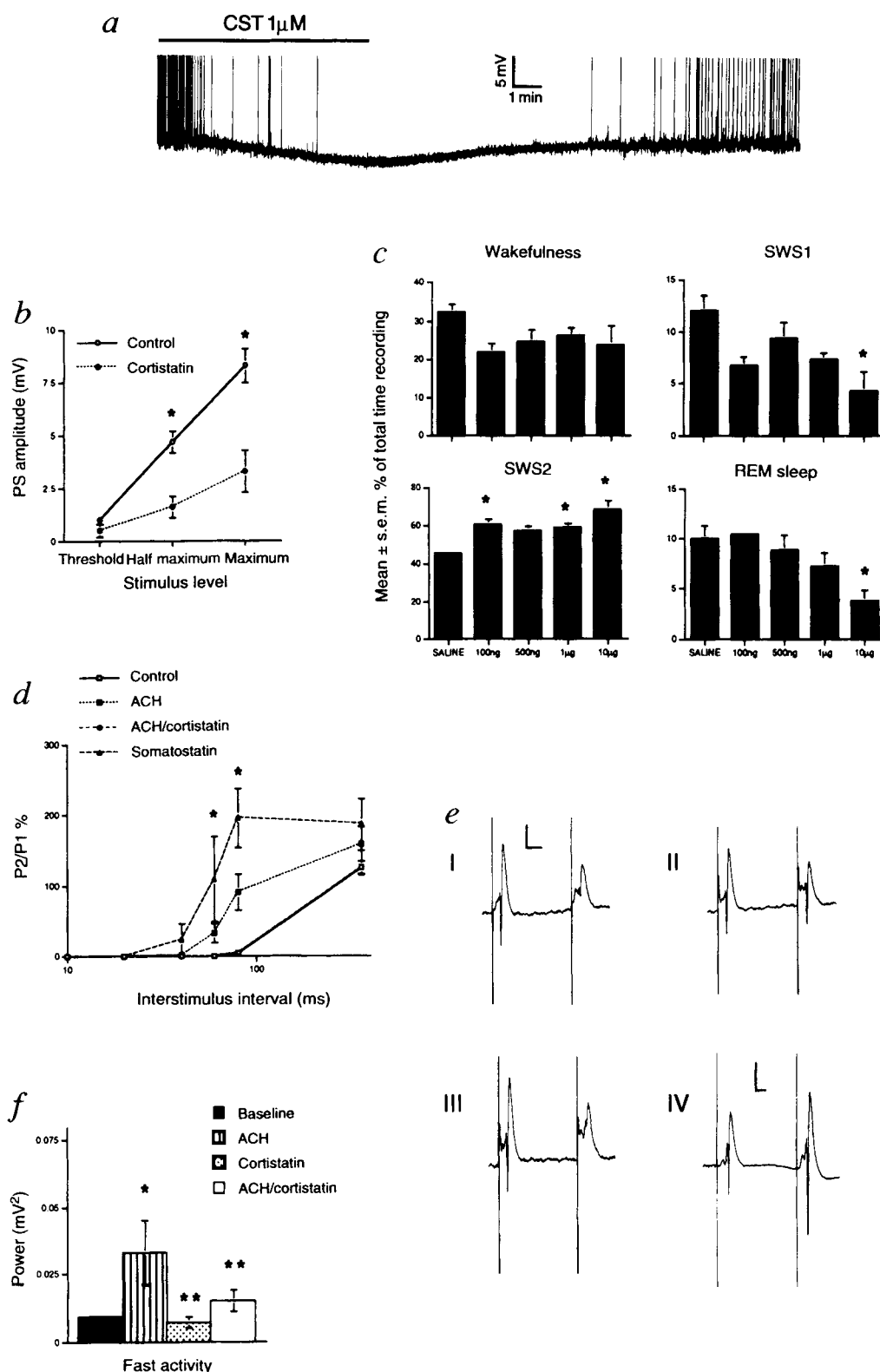
FIG. 3 Cortistatin binds to somatostatin receptors. **a**, Displacement by somatostatin and cortistatin of [¹²⁵I]-somatostatin bound to GH₄ pituitary cells. Cortistatin (filled circles) displaced [¹²⁵I]-somatostatin as effectively as somatostatin (white circles). The combined data from four independent experiments are plotted as mean $\pm$ s.e. TRH and VIP did not displace [¹²⁵I]-somatostatin. **b**, Cyclic AMP stimulation in GH₄ cells. Cultured cells were treated with VIP, TRH, somatostatin and cortistatin, and the intracellular cAMP was measured. VIP and TRH increased the intracellular concentration of cAMP whereas somatostatin or cortistatin did not. **c**, Inhibition of stimulated cAMP levels by somatostatin and cortistatin. Both statins were equally effective in inhibiting cAMP in VIP- or TRH-stimulated cells. **METHODS.** GH₄ cells were grown in MEM medium with 12% fetal calf serum in 6-well plates at 10^6 cells ml⁻¹. Each well then received 10^6 c.p.m. ml⁻¹ of [¹²⁵I]-somatostatin (NEN, DuPont), either alone or with increasing concentrations of somatostatin (Sigma), or cortistatin (95%; purified by reverse-phase HPLC). We included aprotinin and leupeptin (Sigma; 2 μ g ml⁻¹), which reduced nonspecific binding to 20% of total bound radioactivity. We took the binding of [¹²⁵I]-somatostatin in the presence of 10^{-7} M somatostatin to represent nonspecific binding⁶. For cAMP experiments, we grew GH₄ cells under the same conditions as for binding experiments. Cells were washed with MEM without serum, but containing leupeptin, aprotinin and 1 mM 3-isobutylmethylxanthine. Cells were pretreated with somatostatin-14 (Sigma) and cortistatin-14 for 15 min and VIP (10^{-6} M; Bachem) or TRH (10^{-7} M; Calbiochem) was then added. [³H]-cAMP was added to each well before the content was removed, to calculate recovery. For cAMP measurements, we used a radioimmunoassay kit (Amersham) according to the manufacturer's instructions. Each time point represents 2–4 replicates; experiments were done twice.

for saline-treated control animals. We also observed a significant reduction in paradoxical (REM) sleep with the highest dose of cortistatin, in contrast to the reported increase in REM sleep after administration of a similar dose of somatostatin¹¹.

Because acetylcholine is a major regulator of sleep, we tested whether cortistatin produced its effects on sleep, in part, by altering cholinergic activity¹². We first examined the interactions between cortistatin and ACh on hippocampal CA1 neurons using evoked paired-pulse (PP) stimulation to test feed-forward and feedback inhibitory processes mediated in part by hippocampal

FIG. 4 Cortistatin modulates cortical and hippocampal electrophysiology *in vitro* and *in vivo*. **a**, Current-clamp recording of a CA1 neuron manually depolarized to -65 mV (resting membrane potential was -70 mV) to elicit action potential firing (upward deflections, truncated). Superfusion of $1 \mu\text{M}$ cortistatin-14 (bar above record) completely abolishes action potential discharge; the associated hyperpolarization is maximal at 7 min after onset of the response. **b**, Effects of cortistatin on population spike (PS) amplitudes in CA1 neurons *in vivo*. We generated stimulus-response curves and related the PS amplitude monotonically to stimulus intensity tested at three response levels: threshold, half-maximal and maximal (control mean half-maximal PS amplitude, 4.7 ± 0.5 mV; $n = 5$). Microiontophoretic application of cortistatin significantly decreased PS amplitudes in CA1. Asterisks represent significance levels at $P < 0.05$. **c**, Effect of intracerebroventricular administration of cortistatin on the sleep-wake cycle of the rat. Cortistatin increases the period of slow-wave sleep and decreases REM sleep. Asterisks, $P < 0.05$; ANOVA. **d**, Effects of iontophoretically applied ACh (0.9 M), somatostatin (1.5 mM) and cortistatin on PP responses in CA1 neurons *in vivo*. Stimulation intensity was adjusted after drug treatment to achieve control PS amplitudes before PP measurements were made (asterisks, $P < 0.05$). **e**, Representative recordings of field potentials elicited in CA1 by commissural stimulation. In the baseline recordings (I; calibration bars: 2 mV and 10 ms), the second response is completely inhibited at this interstimulus interval (80 ms). Iontophoretic administration of ACh reduced PP inhibition (II). This effect was antagonized by the simultaneous application of cortistatin (III). Somatostatin markedly decreased PP inhibition (IV; calibration bar, 1 mV and 10 ms). **f**, Effects of microiontophoretically (100 – 250 nA) applied ACh and cortistatin on local EEG activity recorded in the visual cortex. ACh markedly increased the averaged EEG power spectra (4 -s epochs over 3 min) in the 8 – 16 -Hz frequency band range. In contrast, the averaged EEG during infusion of cortistatin alone, or of ACh and cortistatin combined, did not differ from baseline. Results from the experimental groups were derived from averaged EEG spectral activity determinations and expressed as means $\pm$ s.e.m. (asterisk, $P < 0.05$ from baseline; two asterisks, $P < 0.05$ from ACh; ANOVA).

METHODS. Recordings were made intracellularly in rat hippocampal slices using sharp glass micropipettes¹⁹. We recorded from 11 hippocampal CA1 pyramidal cells with an average resting membrane potential of -70 ± 1 mV



(mean $\pm$ s.e.m.) and action potential of 103 ± 2 mV. For the *in vivo* studies, male Sprague-Dawley rats were anaesthetized with halothane (0.9 – 1.1%). We stimulated the commissural pathway and elicited field potentials in the CA1 region as described¹⁵. Cortistatin (1 mg ml^{-1}) was dissolved in saline and administered iontophoretically through one barrel of a multibarrelled micropipette. We acquired data with software (LabView Instruments) as described¹⁵. Procedures for recordings and PP studies are described elsewhere^{20,21}.

interneurons^{13,14}. Half-maximal stimulation revealed a characteristic biphasic PP response curve¹⁵. Microiontophoretic application of ACh significantly antagonized the inhibitory phase of PP responses typical of interstimulus intervals of 60–80 ms ($P < 0.05$, Fig. 4d; data from 80-ms interval are shown in Fig. 4e, I–III). Cortistatin itself (not shown) had no significant effects on PP responses in CA1 but completely antagonized the effects of ACh ($P < 0.05$). The effects of somatostatin on PP responses were similar to those of ACh (Fig. 4d; data from 80 ms shown in Fig. 4e, IV). To determine whether cortistatin also interacts with cholinergic systems that regulate cortical function, we investigated the effects of cortistatin on ACh-induced desynchronization of EEG in the cerebral cortex of anaesthetized rats. Slow waves (0.5–4 Hz) dominated the EEG baseline of these rats, whereas iontophoretic administration of ACh desynchronized the local EEG by increasing the potency of the θ (4.5–8 Hz) and β (13–20 Hz) bands. This effect was prevented by simultaneous electrophoretic application of cortistatin (Fig. 4f).

We have described the isolation of a cDNA clone from an mRNA encoding the precursor of a new member of the somatostatin family whose distribution is primarily restricted to GABAergic cortical interneurons. Cortistatin-14 and somatostatin-14 appear to have similar inhibitory effects on the physiology of hippocampal neurons, suggesting that they could act through the same receptors *in vivo*. Cortistatin's effects on the activity of hippocampal neurons *in vivo* and on sleep physiology are, however, distinct from those of somatostatin, so cortistatin might bind differentially to different somatostatin-receptor subtypes from those analysed here, or it could act on μ -opioid receptors like the somatostatin analogue octeotride and the μ -receptor antagonist CTOP¹⁶. The different functional responses may indicate that there is an uncharacterized cortistatin receptor. The lack of effect of cortistatin on PP inhibition contrasts with the potent disinhibitory effect of somatostatin on this measure observed here, which is supported by somatostatin reduction of GABA-mediated synaptic potentials in CA1 pyramidal neurons¹⁷. This is another functional difference between cortistatin and somatostatin. These results are consistent with those from awake rats, in which cortistatin markedly reduces the duration of cortical electrical activity associated with the cholinergic system, as well as the ACh-induced desynchronization of local EEG. Therefore, cortistatin antagonizes the effects of ACh in both the hippocampus and the cerebral cortex *in vivo*. Our findings implicate this novel neuropeptide as a regulator of neuronal activity and sleep. □

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- Shiromani, P. J., Gillin, J. C. & Henriksen, S. J. *Am. Rev. Pharmacol. Toxicol.* **27**, 137–56 (1987).
- Borbély, A. A. & Tobler, I. *Physiol. Rev.* **69**, 605–670 (1989).
- Glushankov, P. et al. *Proc. natn. Acad. Sci. U.S.A.* **81**, 6662–6666 (1984).
- Veber, D. F. et al. *Nature* **280**, 512–514 (1979).
- Eriander, M. G. et al. *Neuron* **7**, 91–100 (1991).
- Schonbrunn, A. H. & Tashjian, A. J. R. *J. Biol. Chem.* **255**, 6473–6483 (1978).
- Halliwel, J. V. & Adams, P. R. *Brain Res.* **250**, 71–92 (1982).
- Moore, S. D. et al. *Science* **239**, 278–280 (1988).
- Schweitzer, P., Madamba, S. & Siggins, G. R. *Nature* **346**, 464–466 (1990).
- Andersen, P., Bliss, T. V. P. & Skrede, K. K. *Exp. Brain Res.* **13**, 208–221 (1971).
- Danguir, J. *Brain Res.* **367**, 26–30 (1986).
- Steriade, M., McCormick, D. A. & Sejnowski, T. J. *Science* **262**, 679–685 (1993).
- Andersen, P., Eccles, J. C. & L  nning, Y. J. *Neurophysiol.* **27**, 607–619 (1964).
- Kandel, E. R. & Spencer, W. A. *J. Neurophysiol.* **24**, 243–259 (1961).
- Steffensen, S. & Henriksen, S. J. *Brain Res.* **538**, 46–53 (1991).
- Maurer, R. et al. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4815–4817 (1982).
- Scharfman, H. E. & Schwartzkroin, P. A. *Brain Res.* **493**, 205–211 (1989).
- deLecea, L. et al. *Molec. Brain Res.* **25**, 286–296 (1994).
- Schweitzer, P. et al. *J. Neurosci.* **13**, 2033–2049 (1993).
- Prospero-Garc  , O., Cn  do, J. R. & Henriksen, S. J. *Pharmac. Biochem. Behav.* **49**, 413 (1994).
- Steffensen, S. C., Campbell, I. L. & Henriksen, S. J. *Brain Res.* **652**, 149 (1994).

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Localization of dopamine D4 receptors in GABAergic neurons of the primate brain

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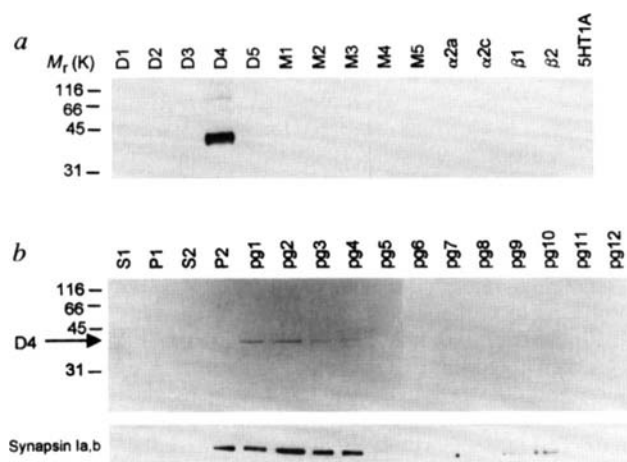
DOPAMINE receptors are the principal targets of drugs used in the treatment of schizophrenia¹. Among the five mammalian dopamine-receptor subtypes, the D4 subtype is of particular interest because of its high affinity for the atypical neuroleptic clozapine^{1–3}. Interest in clozapine stems from its effectiveness in reducing positive and negative symptoms in acutely psychotic and treatment-resistant schizophrenic patients without eliciting extrapyramidal side effects⁴. We have produced a subtype-specific antibody against the D4 receptor and localized it within specific cellular elements and synaptic circuits of the central nervous system. The D4-receptor antibody labelled GABAergic neurons in the cerebral cortex, hippocampus, thalamic reticular nucleus, globus pallidus and the substantia nigra (pars reticulata). Labelling was also observed in a subset of cortical pyramidal cells. Our findings suggest that clozapine's beneficial effects in schizophrenia may be achieved, in part, through D4-mediated GABA modulation, possibly implicating disinhibition of excitatory transmission in intrinsic cortical, thalamocortical and extrapyramidal pathways.

Affinity-purified antibodies selectively bound to a protein of M_r ~40K which was present only in cell lines expressing the dopamine D4 receptor subtype, when examined by western blotting (Fig. 1a). Further, western blot analysis of a subcellular fractionation of the macaque hippocampus revealed D4 antibody reactivity with a ~41K protein present in synapsin-I-enriched membrane fractions (Fig. 1b). The M_r of proteins detected by the antibodies is in good agreement with the predicted size of the D4 receptor polypeptide (~42K)², strongly supporting the specificity of the antibodies for the D4 subtype of DA receptors.

In both the cerebral cortex and hippocampus, D4 receptor immunoreactivity was observed in pyramidal and in non-pyramidal neurons (Fig. 2a–d). These results are consistent with *in situ* hybridization evidence of D4 messenger RNA localization in the cerebral cortex⁵. The expression of D4 receptors in non-pyramidal neurons was notable because dopamine-receptor subtypes examined so far (D1 and D5) localize predominantly to pyramidal neurons both in cerebral cortex and hippocampus⁷. Most non-pyramidal neurons in the cerebral cortex are local circuit neurons or interneurons that release the inhibitory neurotransmitter GABA (γ -aminobutyric acid)⁸. Double-labelling with antibodies against GABA and/or the calcium-binding protein parvalbumin⁹ confirmed that the D4-positive non-pyramidal neurons were indeed GABAergic (Fig. 2e). In addition, electron microscopic analysis revealed asymmetric synapses on the cell bodies (Fig. 3a) and dendritic shafts (Fig. 3c) of the D4-labelled nonpyramidal neurons, a characteristic ultrastructural feature of interneurons. The immunoreaction product was frequently associated with the plasma membrane (Fig. 3a–c), endoplasmic reticulum and cytoplasm. It is not clear why the label associates with the cytoplasmic face of the plasma membrane, but this also occurs with antibodies to the predicted extracellular segments of other neurotransmitter receptors¹⁰. To our knowledge, the localization of D4 receptors in interneurons provides the first evidence of a role for this receptor in the modulation

FIG. 1 Specificity of D4 antibodies. Western blot of purified D4 antibodies (1 mg ml^{-1}) with **a**, crude membrane protein fractions ($5 \mu\text{g}$ per lane) of recombinant Sf9 cell lines expressing human D1 and D2, D3_{rat}, D4.2 (with two 48-nucleotide repeats) and D5 dopaminergic; M1, M2, M3, M4 and M5 muscarinic; $\alpha 2a$, $\alpha 2c$, $\beta 1$ and $\beta 2$ adrenergic; and 5HT1A serotonergic receptors; and **b**, various subcellular fractions ($5 \mu\text{g}$ per lane) of monkey hippocampus: S1 (supernatant 1), S2, P1 (pellet 1), P2, and pg1 to pg12 (Percoll gradient fractions 1 to 12). Bottom panel, hippocampal subcellular fractionation blot reprobed with antibodies to the synaptosomal protein synapsin I. The migration of relative molecular mass markers indicated on the left.

METHODS. To prepare D4-receptor-subtype-specific antibodies, DNA encoding the N terminus (amino-acid residues 1–32) of the human DA D4 receptor² was amplified by polymerase chain reaction (PCR) using chemically synthesized human D4 cDNA²⁸ and the following synthetic oligonucleotide primers: D₄5': 5'-GAGTGGTGC GGTC CGGAATTCATGGGTAA-CAGATCTACTGCA-3'/D₄3': 5'-TTTATGACGACGASCCTCGACACAGCAAGTC-CAGCGGATGC-3'. PCR was carried out with *Pfu* polymerase and *Pfu* buffer 1 (Stratagene) for 35 cycles (1 min at 95 °C, 1 min at 58 °C, 1 min at 72 °C) using a thermocycler (Coy). The PCR product was digested with *Eco*RI and *Sall* and inserted in-frame into pGEX-4T-1 (Pharmacia) and pMalc2 (New England Biolabs) to yield plasmid DNAs encoding a glutathione-S-transferase- and a maltose-binding protein-D4 dopamine-receptor fusion protein, GST-D4 and MBP-D4, respectively. Both constructs were confirmed by dideoxynucleotide chain termination sequencing. GST-D4 and MBP-D4 were induced in *E. coli* strain XL-1 Blue by addition of 1 mM isopropyl thioalgalactoside and purified according to the manufacturer's instructions using glutathione (Pharmacia) and amylose (New England Biolabs) resins, respectively. Three New Zealand White rabbits were immunized with GST-D4 and antibodies reactive with the D4 portion of GST-D4 were affinity-purified on nitrocellulose strips containing MBP-D4 as described⁶. Membrane fractions from recombinant baculovirus-



infected Sf9 cell lines expressing dopaminergic, muscarinic, adrenergic and serotonergic receptor subtypes were a gift from M. Dennis (Biosignal). Soluble and membrane-bound protein fractions of monkey prefrontal cortex, hippocampus and substantia nigra were isolated as described.^{29,30} Proteins were solubilized overnight in 2× loading buffer, fractionated by 12.5% SDS-PAGE, electroblotted according to standard procedures and incubated with affinity-purified human D4 (1 mg ml^{-1}) antibodies or synapsin I antibodies (diluted 1:200) (gift from A. Czernik); after washing, blots were incubated with goat anti-rabbit IgG (Pierce) (1:25,000) and bound antibody detected using an ECL kit (Amersham).

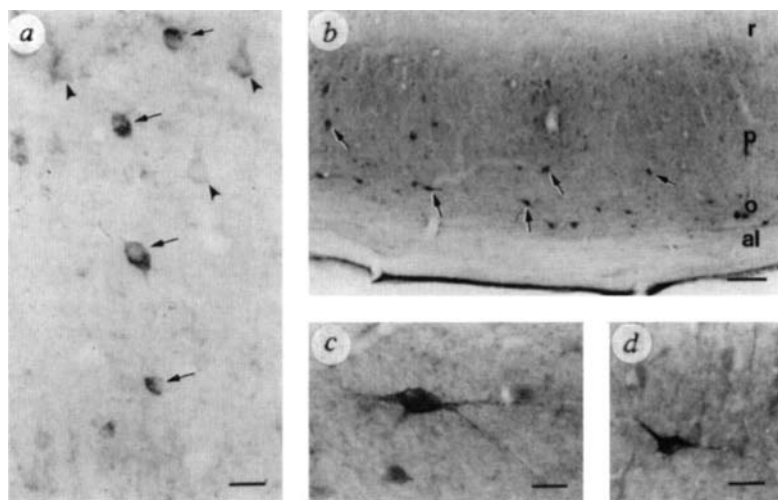


FIG. 2 **a–e**, D4-receptor-positive non-pyramidal neurons (interneurons) in the macaque frontal cortex and hippocampus. **a**, Column of immuno-reactive interneurons (arrows) in layers III–V in macaque premotor cortex (area 6). Arrowheads point to lightly labelled D4-positive pyramidal neurons in the same area. **b**, Low-power magnification of the hippocampal CA1 subfields (al, alveus; o, stratum oriens; p, stratum pyramidale; r, stratum radiatum). Arrows indicate D4-positive non-pyramidal neurons; immunoreactivity in pyramidal neurons is sparse. Most interneurons are localized to the stratum oriens and the lower half of the pyramidal layer; those in the upper half of the pyramidal layer and stratum radiatum are less frequent. **c**, **d**, Higher-power micrograph of D4-receptor-immunoreactive interneurons in the stratum oriens (**c**) and stratum pyramidale (**d**). Scale bars: **a**, 25 μm ; **b**, 100 μm ; **c** and **d**, 20 μm . **e**, Double labelling of neurons in the prefrontal cortex with D4-receptor antibody and parvalbumin. A single-labelled parvalbumin neuron is indicated by an arrowhead (brown) and single-labelled D4 pyramidal neurons by open arrows (blue-grey). The double-labelled neuron (brown and blue-grey) in same neuron; filled arrow provides evidence for a D4 receptor in a subset of GABAergic neurons.

METHODS. Three adult rhesus monkeys (*Macaca Mulatta*), treated in accordance with institutional guidelines, were deeply anaesthetized with Nembutal (100 mg per kg given intravenously) and perfused through the heart with 3–4% paraformaldehyde/0.1% glutaraldehyde/15% picric acid in

0.1 M phosphate buffer. Coronal blocks containing frontal, temporal and parietal lobes, midbrain and cerebellum were cut frozen at 30–40 μm for examination in a light or electron microscope. After incubation with polyclonal D4 antibody (1:100) for 48 h (4 °C), sections were processed using avidin-biotin and biotinylated goat anti-rabbit antibody and an ABC Elite kit (Vector Labs). Bound antibodies were visualized by incubation with 0.05% diaminobenzidine (DAB) and addition of 0.01% H_2O_2 peroxide in phosphate buffer or by DAB-glucose oxidase reaction in the presence of nickel ammonium sulphate. Controls: (1) sections incubated with primary antibody preincubated with D4 fusion protein; (2) sections incubated in preimmune serum; or (3) primary antibody omitted. None yielded immunoreaction product. For double-labelling in **e**, sections were incubated with D4 antibody and monoclonal antibodies against parvalbumin (Sigma) or GABA (Chemicon). Sections were first processed to visualize the D4 antibody yielding a blue-grey product. GABA or parvalbumin antibodies were visualized using DAB-glucose oxidase without nickel ammonium sulphate, giving a brown product distinguishable from the grey-blue-stained D4 receptors.

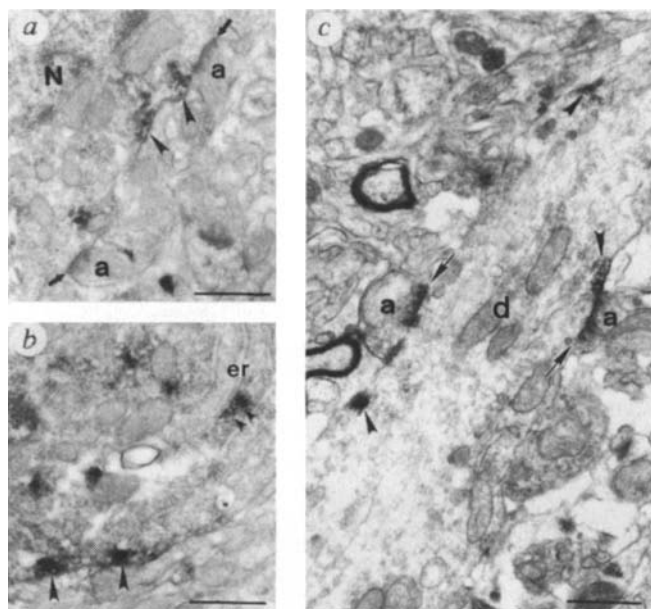


FIG. 3 a–c, High-power electron micrographs featuring a portion of the cell body and a dendrite of a D4 immunoreactive nonpyramidal neuron from area 46. The immunoreaction product in the cell body (a, b) and dendrite (c) is frequently apposed to the cytoplasmic surface of plasma membrane. In the soma (b), this product is also seen in the rough endoplasmic reticulum (er, small arrowheads). Asymmetric synapses on the cell body and dendritic shaft (arrows in a and b) are typical features of a local circuit neuron in the cortex. Micrographs in a and b are of sections not stained with lead citrate. N, nucleus; a, axon; d, dendrite. Scale bars in a–c, 0.5 μ m.

METHODS. For electron microscopy, vibratome sections stained for D4 receptor were treated in 1–2% osmium tetroxide in phosphate buffer and flat-embedded in Durcupan (Fluka). Selected blocks were cut serially into ultrathin sections on an Ultramicrotome (Reichert), stained with lead citrate and uranyl acetate or left unstained and examined with a Jeol transmission electron microscope.

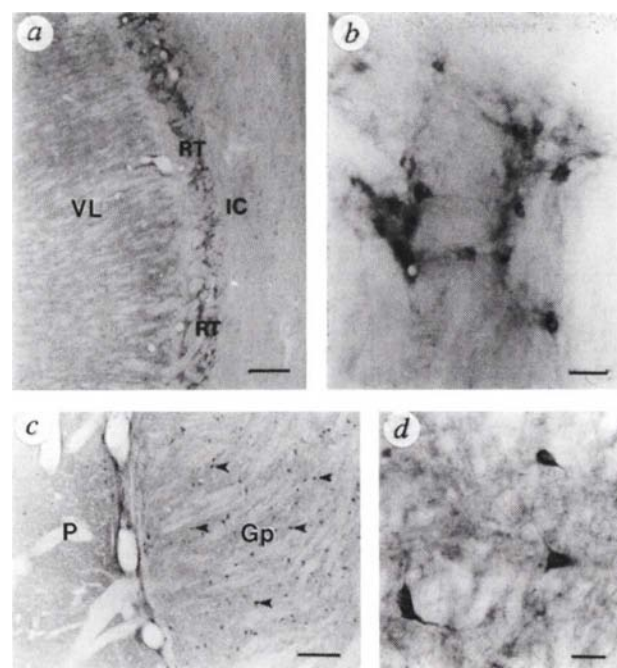


FIG. 4 a, b, Intense immunoreactivity in the thalamic reticular nucleus (RT) at the level of the ventrolateral (VL) thalamic nucleus which, by contrast, showed minimal immunoreactivity. IC, internal capsule. A higher-power micrograph of the RT nucleus is shown in b; strong D4-receptor staining in neurons can be seen. c, d, D4-receptor immunoreactivity in the basal ganglia: c shows a low-power micrograph of putamen (P) and globus pallidus (GP)—immunoreactivity in putamen is negligible; arrowheads indicate intensely D4-labelled neurons in the globus pallidus, shown at higher power in d. Scale bars: a, 0.5 mm; b, 40 μ m; c, 200 μ m; d, 30 μ m.

of GABAergic transmission in the cerebral cortex and hippocampus.

Another potential mechanism for dopamine regulation of GABAergic transmission by D4 receptors was evident in the thalamus. Among thalamic nuclei, D₄ immunoreactivity in the reticular nucleus was particularly prominent (Fig. 4a, b). This nucleus has not previously been considered a target of dopamine input, but it has been found that dopamine axon terminals are distributed throughout this structure (S.M. Williams and P.S. Goldman-Rakic, unpublished observations). As reticular neurons are exclusively GABAergic¹¹, they provide a powerful means for inhibiting thalamocortical activity¹² through their projections to dorsal thalamic nuclei¹³. The effect of clozapine on reticular cell firing is not known, but the present findings suggest that, as a result of its actions on D4 receptors, clozapine may facilitate thalamocortical transmission by regulating the release of GABA from reticular neurons. Indeed, clozapine increases sleep associated with rapid eye movement and induces epileptogenic electroencephalogram abnormalities¹⁴, observations that are consistent with the idea that clozapine acts, in part, to disinhibit the thalamocortical pathway and raise cortical excitability.

Evidence that the D4 receptor participates in modulating GABAergic transmission also comes from extrapyramidal circuits of the basal ganglia (Fig. 4c, d); the basal ganglia circuits have commonly been implicated in the pathophysiology of schizophrenia. We detected D4 receptor immunoreactivity in neurons of both segments of the globus pallidus (Fig. 4c, d) and the pars reticulata of the substantia nigra (results not shown). The immunoreactive neurons in both structures are known to be GABAergic¹⁵. In contrast, labelling of GABAergic neurons in the caudate nucleus and putamen was barely above background (Fig. 4c), in agreement with studies on the localization of D4 mRNA in the striatum^{2,5}. Also, clozapine treatment decreases the level of glutamate decarboxylase mRNA in the globus pallidus, but not in the striatum¹⁶. The distribution of D4 receptors in the basal ganglia is consistent with this effect occurring as a result of the action of clozapine at D4 receptors. Similarly, the relative differences in levels of D4 receptor expression in nuclei of the basal ganglia may be relevant to the failure of atypical neuroleptics like clozapine to produce the debilitating extrapyramidal side-effects in schizophrenia³. The D4-receptor-positive GABAergic neurons of the pars reticulata and the internal segment of the globus pallidus constitute major links in the basal ganglia loop circuits¹⁷ that regulate the motor thalamus and the pyramidal cells of the cerebral cortex in sequence. It remains to be determined

whether D4 antagonists might attenuate GABA release in the nigro-thalamic and/or pallido-thalamic pathways, and so enhance excitatory transmission through the thalamus.

Our results indicate that the action of clozapine may result from direct and indirect effects on pyramidal cell firing. Direct effects could be mediated by blocking D4 receptors on cortical pyramidal cells, where dopamine is inhibitory¹⁸. The indirect effect, namely the disruption of feedforward inhibition on pyramidal cell firing, is potentially a more powerful influence as interneurons have highly divergent inhibitory projections to large numbers of pyramidal neurons¹⁹. This effect is consistent with pharmacological evidence that dopamine inhibits pyramidal cell firing, in part by activation of GABA-inhibitory neurons^{20,21}; clozapine could negate this effect through a reduction of GABA release²². GABA release in the cerebral cortex is modulated by the family of D2-like receptors²³, of which the D4 receptor is a member. Clozapine's net therapeutic efficacy could therefore be to enhance excitatory

transmission in pyramidal cells by releasing them from intracortical and subcortical sources of inhibition. Excess inhibition in cortical circuits would be compatible with reports both of glutamate deficiency²⁴ and hypofrontality²⁵ in schizophrenic patients.

Other neurotransmitter receptors may complement or interact with the D4 receptor to achieve clozapine's effect in subsets of

schizophrenic patients. GABAergic interneurons in the cortex receive a major serotonergic innervation²⁶. As clozapine has a high affinity for serotonergic receptors^{1,3} and inhibits the 5-HT₂-receptor-mediated excitation of interneurons²⁷, a synergistic action of D4 and serotonergic receptors may hold a further clue to the palliative action of clozapine in schizophrenic patients. □

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- Seeman, P. & Van Tol, H. H. M. *Trends pharmac. Sci.* **15**, 264–270 (1994).
- Van Tol, H. H. M. et al. *Nature* **350**, 614–619 (1991).
- Meltzer, H. Y. J. *clin. Psychiat.* **55**, 47–52 (1994).
- Lee, M. A., Thompson, P. A. & Meltzer, H. Y. J. *clin. Psychiat.* **55**, 82–87 (1994).
- Mansour, A. et al. *Soc. Neurosci. Abstr.* **17**, 599 (1991).
- Bergson, C. et al. *J. Neurosci.* **15**, 7821–7837 (1995).
- Levey, A. I. et al. *Proc. natn. Acad. Sci. U.S.A.* **90**, 8861–8865 (1993).
- Houser, C. R., Vaughn, J. E., Hendry, S. H. C., Jones, E. G. & Peters, A. in *Cerebral Cortex Vol. 2* (eds Jones, E. G. & Peters, A.) 63–89 (Plenum, New York and London, 1984).
- Celio, M. R. *Neuroscience* **35**, 375–475 (1990).
- Siegel, S. J. et al. *J. Neurosci.* **15**, 2707–2719 (1995).
- Houser, C. R., Vaughn, J. E., Barber, R. P. & Roberts, E. *Brain Res.* **200**, 341–354 (1980).
- Stenrade, M., McCormick, D. A. & Sejnowski, T. J. *Science* **262**, 679–685 (1993).
- Liu, X.-B., Warren, R. A. & Jones, E. G. *J. comp. Neurol.* **352**, 187–202 (1995).
- Elomaa, E. *Med. Hypoth.* **43**, 245–246 (1994).
- Mugnaini, E. & Oertel, W. H. in *Handbook of Chemical Neuroanatomy Vol. 4* (eds Bjorklund, A. & Hokfelt, T.) 436–608 (Elsevier, Amsterdam, 1985).
- Delfs, J. M., Anegawa, N. J. & Chesselet, M. F. *Neuroscience* **66**, 67–80 (1995).
- DeLong, M. R. & Wichmann, T. *Clin. Neurosci.* **1**, 18–26 (1993).
- Bunney, B. S. & Agajanian, G. K. *Life Sci.* **19**, 1783–1792 (1976).

- Martin, K. A. C. Q. *J. exp. Physiol.* **73**, 637–702 (1988).
- Ferron, A., Thierry, A. M., Le Douarin, C. & Glowinski, J. *Brain Res.* **302**, 257–265 (1984).
- Gellman, R. L. & Aghajanian, G. K. *Brain Res.* **600**, 63–73 (1993).
- Bourdelaïs, A. J. & Deutsch, A. Y. *Cereb. Cortex* **4**, 69–77 (1994).
- Retaux, S., Besson, M. J. & Penit-Soria, J. *Neuroscience* **42**, 61–71 (1991).
- Kim, J. S., Kornhuber, H. H., Schmid-Burgh, W., Holtzmueller, B. *Neurosci. Lett.* **20**, 379–382 (1980).
- Weinberger, D. R., Berman, K. F. & Zec, R. F. *Arch. gen. Psychiat.* **43**, 114–124 (1986).
- DeFelipe, J., Hendry, S. H. C., Hashikawa, T. & Jones, E. G. *Cereb. Cortex* **1**, 117–133 (1991).
- Gellman, R. L. & Aghajanian, G. K. *Neuroscience* **58**, 515–525 (1994).
- Chio, C. L. et al. *J. Biol. Chem.* **269**, 11813–11819 (1994).
- Huttner, W. B., Schiebler, W., Greengard, P. & De Camilli, P. *J. Cell Biol.* **96**, 1374–1388 (1983).
- Dunkley, P. R., Jarvie, P. E., Heath, J. W., Kidd, J. G. & Rostas, J. A. P. *Brain Res.* **372**, 115–129 (1986).

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Regulation by the ribosome of the GTPase of the signal-recognition particle during protein targeting

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THE signal-recognition particle (SRP) is important for the targeting of many secretory and membrane proteins to the endoplasmic reticulum (ER). Targeting is regulated by three GTPases, the 54K subunit of SRP (SRP54), and the α - and β -subunits of the SRP receptor¹. When a signal sequence emerges from the ribosome, SRP interacts with it and targets the resulting complex to the ER membrane by binding to the SRP receptor. Subsequently, SRP releases the signal sequence into the translocation channel^{2,3}. Here we use a complex of a ribosome with a nascent peptide chain, the SRP and its receptor, to investigate GTP binding to SRP54, and GTP hydrolysis. Our findings indicate that a ribosomal component promotes GTP binding to the SRP54 subunit of SRP. GTP-bound SRP54 is essential for high-affinity interaction between SRP and its receptor in the ER membrane. This interaction induces the release of the signal sequence from SRP, the insertion of the nascent polypeptide chain into the translocation channel, and GTP hydrolysis. The contribution of the ribosome had previously escaped detection because only synthetic signal peptides were used in the analysis⁴.

To determine the role of GTP in protein targeting to the ER, we investigated GTP binding to SRP54 using crosslinking by ultraviolet light^{4,5} in a system consisting of [α -³²P]GTP, purified SRP and purified ribosome–nascent chain complexes (RNC). The nascent chains consisted of the first 86 amino acids of the secretory

protein preprolactin (PPL86). We found that [α -³²P]GTP binding to SRP54 was dependent on the presence of RNC^{PPL86} (Fig. 1a). SRP alone bound GTP only weakly (Fig. 1a). We conclude that RNCs stimulate GTP binding to SRP54. Earlier, synthetic signal peptides were found to inhibit binding of guanine nucleotides to SRP54 (refs 1, 4). Our results show that the RNC overcomes this inhibition.

To determine whether it is a ribosomal component or the signal sequence that stimulates GTP binding to SRP54, GTP crosslinking to SRP54 was tested in the presence of ribosomes carrying either an authentic or a non-functional, mutated signal sequence (RNC^{PPL86} or RNC^{PPL86}mut, respectively), or lacking nascent chains. In the presence of RNC^{PPL86}, stimulation of GTP crosslinking was half-maximal at 7.5 nM SRP, with saturation at ~30 nM (Fig. 1b). RNC^{PPL86}mut or wheat-germ ribosomes lacking nascent chains also stimulated GTP crosslinking, but less efficiently than RNC^{PPL86} (Fig. 1b). We conclude that a ribosomal component is sufficient for stimulating GTP binding to SRP54. To investigate the additional effect of the signal sequence on stimulating GTP binding, we determined the affinity of SRP for GTP in the presence of either RNC^{PPL86} or RNC^{PPL86}mut. Figure 1c shows that the affinity of SRP for GTP is the same in both cases, but the maximum number of GTP molecules bound to SRP54 (B_{max}) is different. We conclude that the presence of a signal sequence on a nascent chain increases the binding of SRP to the ribosome, and as a result more GTP molecules can be bound by the increased number of ribosome-associated SRP molecules.

Increased binding of GTP can be brought about by a decrease in the affinity for GDP or increased affinity for GTP. To test this, [α -³²P]GDP crosslinking to SRP54 was used to compete with increasing concentrations of unlabelled GTP or GDP. Half-maximal inhibition by unlabelled GTP was seen with RNC^{PPL86}–SRP at a roughly 10-fold lower concentration than with SRP alone (Fig. 1d), indicating that the association with the ribosome increases the affinity of SRP54 for GTP. In contrast, competition by GDP was the same in the presence or absence of the RNC^{PPL86} (Fig. 1d), indicating that RNCs do not change the affinity of SRP54 for GDP. The affinity of SRP for GDP seems to be ~40-fold higher than that for GTP (Fig. 1d). At physiological concentrations of GTP (~10⁻⁴ M) and GDP (~10⁻⁵ M)^{6,7}, about 70–80% of the free SRP is estimated to be associated with GDP,

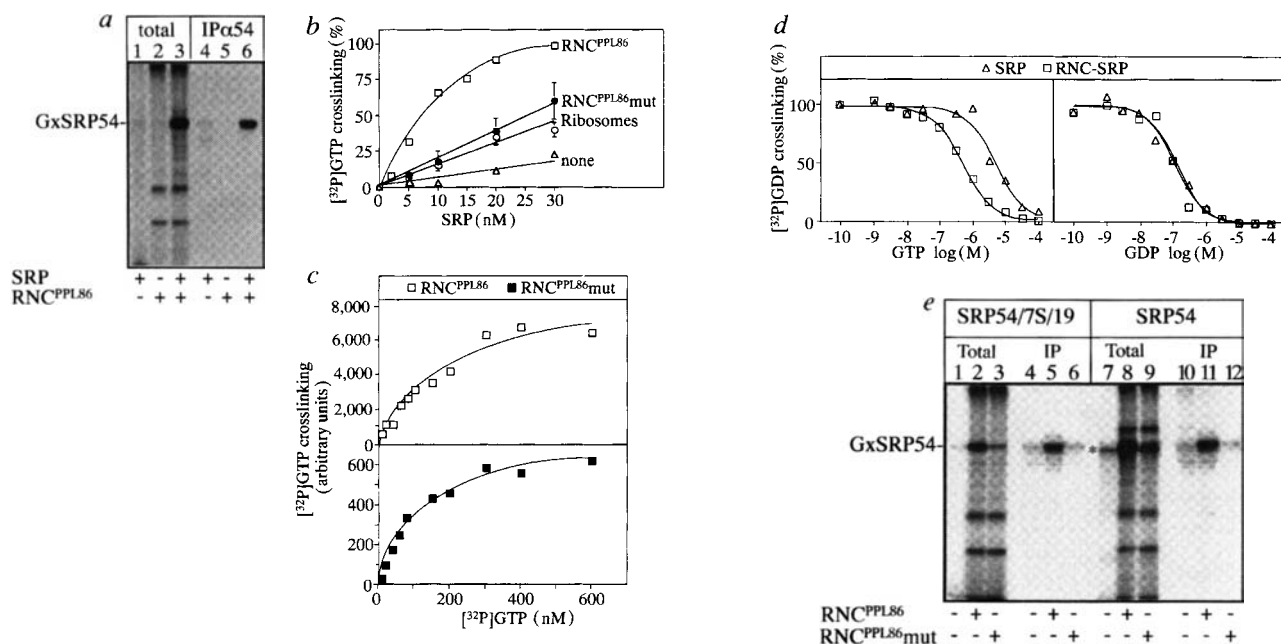


FIG. 1 GTP binding to SRP54. **a**, Crosslinking of radiolabelled GTP to SRP in the presence of RNC^{PPL86}. One third of the samples were directly analysed by SDS-PAGE (lanes 1–3). GxSRP54 represents crosslinked product; two-thirds were immunoprecipitated (IP) under denaturing conditions using an antiserum against SRP54 (lanes 4–6). **b**, Crosslinking of radiolabelled GTP with SRP (Δ), or with SRP in the presence of RNC^{PPL86} ($\square$), RNC^{PPL86mut} ($\bullet$) or wheat-germ ribosomes ($\circ$). GTP crosslinking with 30 nM SRP in the presence of RNC^{PPL86} is taken as 100%. RNC^{PPL86mut} consists of PPL86 containing a non-functional, mutated signal sequence²¹. Values are means $\pm$ s.d. of 3 experiments with RNCmut and wheat-germ ribosomes. **c**, Crosslinking of [α -³²P]GTP to SRP in the presence of either RNC^{PPL86} ($\square$) or RNC^{PPL86mut} ($\blacksquare$). The apparent K_D for GTP in the presence of RNC^{PPL86} or RNC^{PPL86mut} was $0.20 \pm 0.04 \mu\text{M}$ or $0.11 \pm 0.02 \mu\text{M}$, respectively. The relative B_{max} for GTP was 12 times higher with RNC^{PPL86} than with RNC^{PPL86mut}. **d**, Affinity of SRP for GTP. [α -³²P]GDP crosslinking to SRP (Δ) or to RNC-SRP complexes ($\square$) is competed for by increasing amounts of unlabelled GTP (left) or GDP (right). The apparent K_i values for GTP without or with RNC^{PPL86} were $2 \mu\text{M}$ and $0.2 \mu\text{M}$, respectively; K_i for GDP with or without RNC^{PPL86} was $0.05 \mu\text{M}$. **e**, Crosslinking of radiolabelled GTP to SRP54/7S/19 or to SRP54 in the presence or absence of RNCs. Samples were analysed directly (lanes 1–3, 7–9) or after immunoprecipitation of SRP54 (lanes 4–6, 10–12). A 51K protein contaminant from the SRP54 preparation (asterisk) was also labelled with [α -³²P]GTP (lanes 7–9) but was not immunoprecipitated by anti-SRP54 antibodies (lanes 10–12).

METHODS. Truncated transcripts encoding the 86 N-terminal amino-acids of preprolactin (PPL86) were translated in a wheat-germ extract containing non-labelled amino acids to create a ribosome nascent chain complex (RNC^{PPL86})²². After translation of PPL86 mRNA, samples were adjusted to 0.5 M CH₃COOK. Insoluble material was removed by centrifugation (for

5 min at 17,000g). RNC^{PPL86} was pelleted twice (40 min at 400,000g; TLA100.2, Beckman) through a 1 M sucrose cushion containing HMDC buffer (50 mM HEPES-KOH, pH 7.9, 5 mM magnesium acetate, 2 mM DTT, 1 mM cycloheximide) and 0.5 M CH₃COOK. RNC-SRP complexes were obtained by incubation of RNC^{PPL86} with 50 nM SRP after the first centrifugation. Pellets were resuspended in HMDC buffer containing 100 mM CH₃COOK. Wheat-germ ribosomes were purified as RNCs from a wheat-germ translation mix without added mRNA. RNC hydrolysed $\sim 30\%$ of the GTP present during GTP crosslinking. To reduce this hydrolysis (~ 30 -fold), we treated RNCs or wheat-germ ribosomes with 20 mM *N*-ethylmaleimide (NEM) for 15 min at 25 °C and then with 20 mM DTT (data not shown). NEM treatment of RNC^{PPL86} did not affect binding of the nascent chain to SRP or its release from SRP and insertion into the ER (data not shown). But GTP crosslinking to SRP was also stimulated by RNCs and wheat-germ ribosomes without NEM treatment. SRP54 expressed in insect cells and subparticles containing SRP54 were purified as described¹², as was SRP²³. GTP-crosslinking reactions (10–20 μl) containing 2.8 OD₂₆₀ units ml⁻¹ (b–d), 8.4 OD₂₆₀ units ml⁻¹ (a, e) RNCs (RNC-SRP, d) and/or 25 nM (a, e), 10 nM (c), or 20 nM (d) SRP or 25 nM SRP subunits were preincubated in HMDC containing 150 mM potassium acetate for 30 min on ice; 0.5 μM [α -³²P]GTP (3,000 Ci mmol⁻¹; Amersham) was then added. After incubation for 10 min at 25 °C, samples were placed on ice and subjected to UV irradiation for 10 min (ref. 4) and analysed by immunoprecipitation with antiserum against SRP54 (ref. 12) and SDS-PAGE. [α -³²P]GTP-labelled SRP54 was quantified using a PhosphorImager. Curves connecting data points, the apparent inhibition (K_i) and dissociation constants (K_D) were calculated using the nonlinear regression programme GraphPad Prism (GraphPad).

whereas the ribosome-bound form of SRP would be mainly GTP-bound. The exchange of the SRP-bound nucleotide at the ribosome-associated SRP is probably achieved by rapid dissociation of GDP from SRP⁸.

To determine which components of SRP are required for stimulating GTP binding to SRP54, we tested free SRP54 and an SRP subparticle consisting of SRP 7S RNA, and the SRP54 and SRP19 proteins (SRP54/7S/19) in GTP crosslinking experiments. Authentic RNC^{PPL86} stimulated the binding of GTP to both SRP54/7S/19 and SRP54, indicating that the ribosomal interaction with free SRP54 is sufficient for stimulating GTP binding (Fig. 1e). RNC^{PPL86mut} did not stimulate GTP binding either to free SRP54 or to the SRP subparticle (Fig. 1e). The low affinity of SRP54 for the ribosome in the absence of a signal sequence prevents the ribosome from interacting with SRP54 and stimulating GTP binding.

To determine whether GTP binding to SRP54 is required for

the interaction of SRP with its receptor in the ER membrane, we used a mutant SRP54 (SRP54^{N251}) substituted in one of the consensus sequences required for GTP binding (Fig. 2a). A similar mutation in the GTP-binding site of the EF-Tu elongation factor protein reduces GTP affinity⁹. We also found that guanine nucleotides did not bind to SRP subparticles if they contained SRP54^{N251}, whereas they did if the particle contained authentic SRP54 (Fig. 2b). When these particles were tested for binding to microsomal vesicles containing SRP receptor, a 10-fold higher GMP-PNP concentration was required for binding of SRP54^{N251}/7S/19 to microsomes than for binding of SRP54/7S/19 (Fig. 2c). This suggests that SRP54 must be in the GTP-bound state in order to interact tightly with the SRP receptor. GTP binding to SRP54 was also necessary for release of the signal sequence from SRP at the ER membrane. We found that 1–3 μM GMP-PNP reduced the intensity of crosslinking of the signal sequence to SRP or to the authentic subparticle by 50%, but that a ~ 10 -fold higher

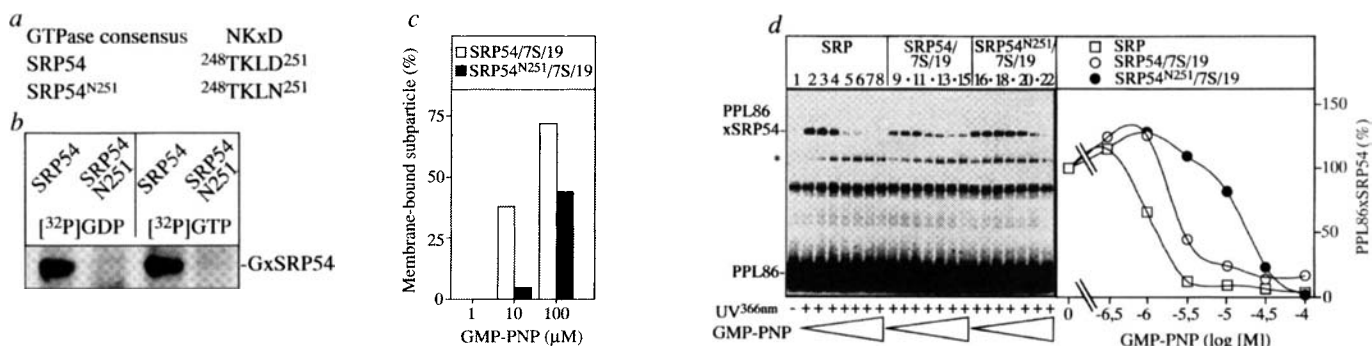


FIG. 2 Functional interaction of SRP with its receptor. **a**, Alignment of the GTP binding consensus sequence NKxD (ref. 7) of SRP54 with the SRP54 GTP-binding-site mutant (SRP54^{N251}). **b**, Crosslinking of radiolabelled guanine nucleotides to RNC^{PPL86}-SRP54/7S/19 and to RNC^{PPL86}-SRP54^{N251}/7S/19. **c**, Binding of SRP54/7S/19 and SRP54^{N251}/7S/19 to rough-ER microsomes treated with puromycin and high salt (PKRMs) at increasing concentrations of the non-hydrolysable GTP analogue GMP-PNP. **d**, GMP-PNP-dependent release of the PPL86 signal sequence from SRP, SRP54/7S/19 and SRP54^{N251}/7S/19. PPL86 nascent chain was crosslinked to SRP, unlabelled SRP54/7S/19 or SRP54^{N251}/7S/19 in the presence of PKRMs and increasing amounts of GMP-PNP. The amount of PPL86 crosslinked to SRP54 was quantified (right). Asterisk denotes the crosslinked product of PPL86 and a membrane component (probably the TRAM protein¹¹).

METHODS. Oligonucleotide-directed site-specific mutagenesis: SRP54 was mutated as described²⁴ using a site-directed mutagenesis kit (Amersham) and the 27-mer oligonucleotide TTGTCATGGCCATTAAGTTTGTCACT. SRP54 and SRP54^{N251} were synthesized in a wheat-germ system (150 μl) in the presence of [³⁵S]methionine (**c**) or unlabelled methionine (**d**). Ribosomes

and insoluble material were removed by centrifugation. SRP subparticles (SRP54/7S/19 and SRP54^{N251}/7S/19) were assembled and purified over a 2-ml 5–20% sucrose gradient as described¹². For further purification, subparticles were incubated with RNC^{PPL86} and RNC^{PPL86}-bound subparticles were sedimented as for Fig. 1. In **b**, reactions (10 μl) containing RNC-SRP subparticles (5.6 OD units ml⁻¹) with 0.3 μM [³²P]GDP (Amersham) or 0.5 μM [³²P]GTP were crosslinked as for Fig. 1. In **c**, mixtures (25 μl) containing SRP54/7S/19 or SRP54^{N251}/7S/19 (4 μl gradient fraction), PKRMs (0.2 equivalents per μl) and GMP-PNP were incubated and centrifuged as described¹². The amount of membrane-bound subparticles obtained in the presence of GDP was subtracted from that obtained in the presence of GMP-PNP. **d**, PPL86 mRNA was translated in a wheat-germ extract containing [³⁵S]methionine and lysyl-tRNA modified at its ε-amino group with the photoactivatable crosslinker 4-(3-trifluoromethylidiazirino)-benzoic acid (ε-TDBA-Lys-tRNA) as described^{25,26}. Reactions (10 μl) contained 20 nM SRP or unlabelled subparticles (6 μl gradient fraction), purified RNC (0.8 OD₂₆₀ ml⁻¹) and PKRMs (0.2 equivalents per μl) as described¹². PKRMs were obtained by treatment of rough microsomes with puromycin and high salt as described¹².

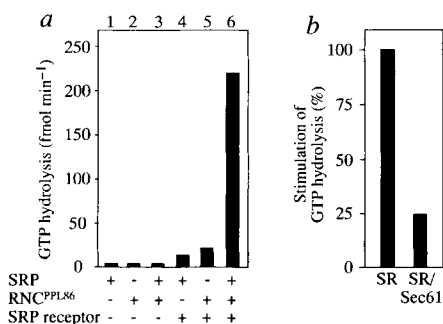
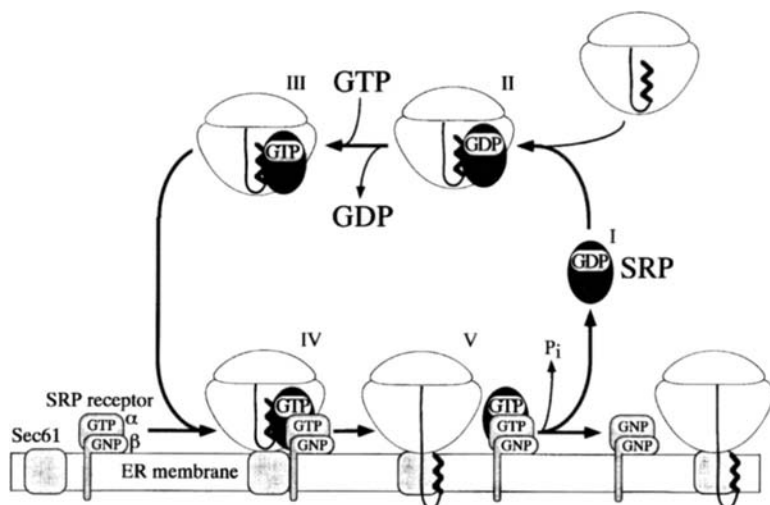


FIG. 3 GTP hydrolysis induced by the interaction between SRP and SRP receptor. **a**, GTP hydrolysis in the presence of SRP, RNC^{PPL86} and proteoliposomes containing SRP receptor (SR). **b**, Stimulation of GTP hydrolysis in the presence of proteoliposomes containing SRP receptor and the translocation channel, Sec61p complex. GTP hydrolysis in the presence of SRP, RNC^{PPL86} and SRP receptor were taken as 100%.

METHODS. Reactions in HMDC buffer containing 0.15 M potassium acetate (5 μl) containing 20 nM SRP and/or RNC^{PPL86} (8.4 OD₂₆₀ ml⁻¹) and/or proteoliposomes containing SRP receptor (25 nM) were preincubated for 20 min at 0 °C. Reactions with proteoliposomes containing SRP receptor (25 nM) or SRP receptor and Sec61p complex (25 nM SR, 0.3 eq μl⁻¹) were incubated with 2.8 OD₂₆₀ ml⁻¹ RNC^{PPL86} and 20 nM SRP. After addition of 2 μM [³²P]GTP (1,500 Ci mmol⁻¹; Amersham) (**a**)/1 μM [³²P]GTP (**b**) samples were incubated for 20 min at 25 °C. Aliquots of the samples were spotted onto polyethyleneimine cellulose thin-layer plates; inorganic phosphate was resolved from GTP using 0.75 M KH₂PO₄, pH 3.3, as solvent. Radioactive TLC spots were quantified using a PhosphorImager (Fujii). Reconstituted vesicles containing SRP receptor or SRP receptor and Sec61p complex were prepared as described²⁰.

FIG. 4 Proposed model of the GTPase cycle of SRP in protein targeting to the ER. SRP is activated by the ribosome to interact with the SRP receptor in the ER membrane. Subsequently the nascent polypeptide chain is released from SRP and inserts into the translocation channel. SRP54 hydrolyses GTP and dissociates from its receptor to engage in a new round of protein targeting. GNP: GTP or GDP.



GMP-PNP concentration was necessary to reduce crosslinking to the mutant subparticle to the same extent (Fig. 2d). The GMP-PNP-dependent release of the signal sequence from SRP leads to membrane insertion of the nascent chain, as judged by the appearance of crosslinks between the nascent chain and membrane components^{10,11}. We and others have observed similar crosslinking in the absence of GMP-PNP due to SRP-independent membrane insertion¹²⁻¹⁴.

Previously it has been shown that the α -subunit of the SRP receptor needs to be GTP-bound for a functional complex to form between SRP and its receptor¹⁵. Together with our results, this suggests that GTP binding to both GTPases controls the docking of the RNC-SRP complex at the ER membrane and release of signal sequence from SRP.

Based on binding experiments with RNC-SRP complexes and ER membranes, it has been suggested that protein targeting to the ER does not require guanine nucleotides⁸. Our results indicate that, on the contrary, without GTP or GMP-PNP, interaction of RNC-SRP with the ER membrane is not functional. Furthermore, the GTP-independent interaction is sensitive to high salt¹⁶ and thus of low affinity.

GTP hydrolysis is stimulated if SRP combines with its receptor under low-salt conditions^{17,18}. However, we found that at physiological salt concentrations (150 mM), GTP hydrolysis was only stimulated if all three components, namely RNC^{PPL86}, SRP and proteoliposomes containing SRP receptor, were combined (Fig. 3a). Proteoliposomes containing SRP receptor and the translocation channel consisting of the Sec61p complex stimulated GTP hydrolysis less (Fig. 3b). One explanation for this could be that SRP receptor alone can induce cycles of signal-sequence release from SRP¹⁹, rebinding of signal sequence, GTP loading, contact with SRP receptor, and GTP hydrolysis. In the presence of the translocation channel, the nascent polypeptide chain would be transferred into it²⁰.

GTP binding to SRP54 at low salt can also be stimulated by the SRP receptor⁴. Under our conditions, the receptor barely stimulates GTP binding to free SRP not to SRP bound to RNC^{PPL86} (data not shown). Thus stimulation of GTP hydrolysis does not result from a SRP receptor-mediated increase of GTP binding to SRP54.

Our results indicate that the ribosome is crucial for the GTPase cycle of the SRP. In our model, free SRP preferentially binds GDP (I in Fig. 4); when SRP-GDP binds to the signal sequence and to the ribosome (II in Fig. 4), a ribosomal component promotes GTP binding to SRP54 (III in Fig. 4); activated SRP-GTP can then engage in a high-affinity interaction with the SRP receptor in the ER membrane (IV in Fig. 4). The tight association of SRP with its receptor requires the SRP and the α -subunit of its receptor to be in the GTP-bound form¹⁵. The signal sequence is then released from SRP54, the nascent chain is inserted into the translocation channel of the ER (V in Fig. 4), and SRP and its receptor dissociate from the translocation channel as a stable complex¹⁸. The receptor-induced GTP hydrolysis by SRP54 (ref. 4) leads to the dissociation of SRP from its receptor¹⁸ (I in Fig. 4): SRP-GDP is now free to engage in a new round of targeting and translocation.

The GTPase cycle of SRP involving the ribosome as a key component provides a regulatory link between translation and translocation machineries. □

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- Walter, P. & Johnson, A. E. *Rev. Cell Biol.* **10**, 87-119 (1994).
- Rapoport, T. A. *Science* **258**, 931-936 (1992).
- Lütcke, H. *Eur. J. Biochem.* **228**, 531-550 (1995).
- Miller, J. D., Wilhelm, H., Gierasch, L., Gilmore, R. & Walter, P. *Nature* **366**, 351-354 (1993).
- Nath, J. P., Eagle, G. R. & Himes, R. H. *Biochemistry* **24**, 1555-1560 (1985).
- Grand, R. J. & Owen, D. *Biochem. J.* **279**, 609-631 (1991).
- Bourne, H. R., Sanders, D. A. & McCormick, F. *Nature* **349**, 117-127 (1991).
- Rapiejko, P. J. & Gilmore, R. *Molec. Biol. Cell* **5**, 887-897 (1994).
- Hwang, Y.-W. & Miller, D. L. *J. Biol. Chem.* **262**, 13081-13085 (1987).
- Görlich, D., Prehn, S., Hartmann, E., Kalies, K.-U. & Rapoport, T. A. *Cell* **71**, 489-503 (1992).
- Görlich, D., Hartmann, E., Prehn, S. & Rapoport, T. A. *Nature* **357**, 47-52 (1992).
- Hauser, S., Bacher, G., Dobberstein, B. & Lütcke, H. *EMBO J.* **14**, 5485-5493 (1995).

- Jungnickel, B. & Rapoport, T. A. *Cell* **82**, 261-270 (1995).
- Lauring, B., Sakai, H., Kreibich, G. & Wiedmann, M. *Proc. natn. Acad. Sci. U.S.A.* **92**, 5411-5415 (1995).
- Rapiejko, P. J. & Gilmore, R. J. *Cell Biol.* **117**, 493-503 (1992).
- Connolly, T. & Gilmore, R. J. *Cell Biol.* **103**, 2253-2261 (1986).
- Connolly, T. & Gilmore, R. J. *Cell Biol.* **123**, 799-807 (1993).
- Connolly, T., Rapiejko, P. J. & Gilmore, R. *Science* **252**, 1171-1173 (1991).
- Connolly, T. & Gilmore, R. *Cell* **57**, 599-610 (1989).
- Görlich, D. & Rapoport, T. A. *Cell* **75**, 615-630 (1993).
- Luirink, J. et al. *Nature* **359**, 741-743 (1992).
- Stueber, D., Ibrahim, I., Cutler, D., Dobberstein, B. & Bujard, H. *EMBO J.* **3**, 3143-3148 (1984).
- Walter, P., Ibrahim, I. & Blobel, G. J. *Cell Biol.* **91**, 545-550 (1981).
- Nakamaye, K. L. & Eckstein, F. *Nucleic Acids Res.* **14**, 9679-9698 (1986).
- Wiedmann, M., Kurzchalia, T. V., Bielka, H. & Rapoport, T. A. *J. Cell Biol.* **104**, 201-208 (1987).
- Hign, S., Görlich, D., Wiedmann, M., Rapoport, T. A. & Dobberstein, B. *J. Cell Biol.* **113**, 35-44 (1991).

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Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing

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TRANSCRIPTIONAL silencing of the *HM* mating-type loci in the yeast *Saccharomyces cerevisiae* is caused by the localized formation of an altered chromatin structure, analogous to heterochromatin in higher eukaryotes. Silencing depends on *cis*-acting sequences, termed silencers, as well as several *trans*-acting factors, including histones H4 and H3, proteins RAP1 and ABF1, and the four SIR proteins (SIR1-4)¹. Each of the four *HM* silencers contains an autonomously replicating sequence (ARS) to which the origin replication complex (ORC)² binds. This six-protein complex is required for initiation of DNA replication, as well as for silencing³⁻⁷. Efficient establishment of the silenced state requires both passage through the S phase of the cell cycle⁸ and SIR1 protein^{9,10}. Previous experiments suggested that SIR1 might be localized to the silencers by binding to ORC and/or RAP1 (ref. 11). Here we report that SIR1 can bind directly to ORC1, the largest of the ORC subunits, and that targeting of SIR1 to ORC1 at a silencer is sufficient to establish a silenced state.

To search for proteins that bind to SIR1, we used a recently described version¹² of the two-hybrid system¹³. As a target we used the carboxy-terminal half of SIR1, from amino acids 346 to 678. This hybrid was expressed at a much greater level than the full-length hybrid protein and still partly complemented a *sir1* mutant (M. Swanson and R.S., unpublished data). Two clones were isolated from a yeast library that interacted strongly and specifically with this SIR1 hybrid. DNA sequencing showed that both plasmids encoded in-frame fusions of the GAL4 activation domain to the amino terminus of the recently described largest subunit of ORC, ORC1 (ref. 14). Both fusions began at amino acid 5 of ORC1 and ended at amino acids 267 and 268, respectively (Fig. 1). This is the region of ORC1 that is important for silencing¹⁴, and is the region most similar to the silencing protein SIR3, with 50% identity within the first 214 amino acids¹⁴. An ORC1 hybrid corresponding closely to the region with highest homology to SIR3 (amino acids 5-228) interacted with SIR1 as strongly as did the two original clones, but shorter N-terminal truncations of ORC1 failed to give a positive signal (Fig. 1).

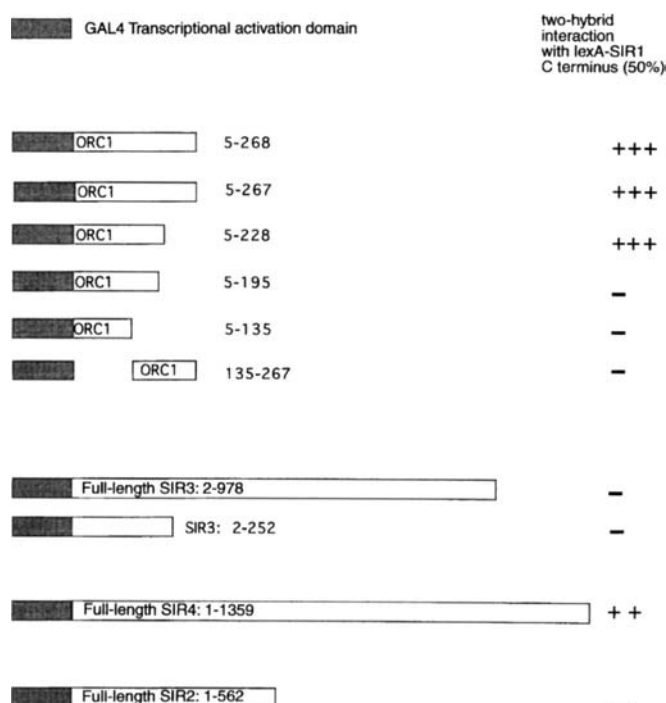


FIG. 1 SIR1 interacts with ORC1 and SIR4 in the two-hybrid system. Shown are various hybrid proteins fused to the GAL4 activation domain (GAD) that were tested against lexA-SIR1 (amino acids 346–678). The strength of the transcriptional activation of the *lacZ* reporter gene is indicated (+ + +, cells turned blue with X-gal indicator in 15–30 min; + +, cells turned blue in 2–4 h; –, no detectable colour within 16 h).

METHODS. The ORC1 hybrids encoding amino acids 5–267 and 5–268 were obtained by screening a yeast genomic GAD library by using a version of the two-hybrid system described recently¹². We constructed the other hybrids.

Because of the homology between SIR3 and ORC1, we tested whether SIR1 would interact with SIR3 in the two-hybrid system. No evidence for an interaction was seen, using either full-length, or just the first 252 amino acids of, SIR3 (Fig. 1). At present we do not understand the significance of the similarity between ORC1 and SIR3.

To assess whether the interaction between SIR1 and ORC1 observed in the two-hybrid system was a direct one, it was tested in reporter strains carrying mutations in *sir1*, *sir2*, *sir3* or *sir4*. The two-hybrid signal was unaffected by any of these mutations, so we conclude that the interaction does not require endogenous SIR1, SIR2, SIR3 or SIR4 proteins.

As a further demonstration that SIR1 bound directly to ORC1 a glutathione *S*-transferase (GST)–SIR1 fusion protein (amino acids 346–678) was expressed in *Escherichia coli* and precipitated from an extract with glutathione-conjugated Sepharose beads. GST–SIR1 bound to glutathione–Sepharose was mixed with ³⁵S-labelled ORC1 (amino acids 5–268) synthesized *in vitro*, and the reprecipitated sample electrophoresed on an SDS–polyacrylamide gel to assess ORC1 binding to SIR1. The labelled ORC1 coprecipitated with GST–SIR1, but did not coprecipitate with GST alone (Fig. 2). Quantitative densitometry showed that there was at least 30-fold greater binding of the ORC1 fragment to GST–SIR1 than to GST alone. In addition, a shorter ORC1 fragment (amino acids 5–195) that did not interact with SIR1 in the two-hybrid system (Fig. 1) also failed to show significant interaction with GST–SIR1 *in vitro* (Fig. 2).

To identify other proteins that bind to SIR1, the silencing proteins SIR4 and SIR2 were tested against the same SIR1 hybrid in the two-hybrid system. Full-length SIR4 was found to interact strongly with SIR1 (Fig. 1), but hybrids containing SIR2,

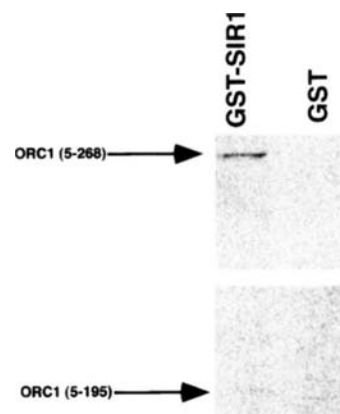


FIG. 2 ORC1 binds to GST1–SIR1 *in vitro*. GST1–SIR1 or GST was mixed with ³⁵S-labelled ORC1 (amino acids 5–268, top) or with ³⁵S-labelled ORC1 (amino acids 5–194, bottom) and precipitated with glutathione–Sepharose beads. The precipitated proteins were subjected to SDS–PAGE. An autoradiogram of the gel is shown. The positions of the ³⁵S-labelled ORC1 fragments are indicated by arrows.

METHODS. SIR1 (amino acids 346–678) was fused to GST in the expression vector, pGEX3. The fusion protein and GST alone were expressed in *E. coli*, and cells lysed by freezing and thawing followed by sonication. GST–SIR1 and GST were purified from the *E. coli* extracts by precipitation with glutathione-conjugated Sepharose beads. A DNA fragment encoding ORC1 amino acids 5–268 was cloned into the T7 expression vector, pET-3c (ref. 17). T7 RNA polymerase was used to make ORC1 RNA from this plasmid. The RNA was incubated for 2 h at 30 °C in a rabbit reticulocyte lysate translation system (Promega) containing ³⁵S-methionine. Labelled ORC1 (8 μl of the lysate) was mixed with ~7 μg of GST or GST–SIR1 bound to glutathione–Sepharose beads in 1 ml phosphate-buffered saline (PBS; 136 mM NaCl, pH 7.4). After 45 min incubation at 4 °C, the beads were washed 4 times with PBS (650 mM NaCl, pH 7.4) and then boiled in SDS loading buffer. The solubilized proteins were subjected to SDS–PAGE. The gel was fixed, soaked in AutoFluor liquid scintillant (National Diagnostics), dried and exposed to X-ray film.

as well as various C-terminal fragments of SIR4, failed to interact (data not shown). Thus the SIR1-interacting domain of SIR4 appeared to be within the N-terminal 60%; it has not been delineated further. Another demonstration that SIR1 interacts with SIR4 is that the LexA–SIR1 hybrid used in these experiments weakly activates the two-hybrid reporter gene in a *sir4* mutant strain, whereas no activation is seen in a *SIR*⁺ strain. Our interpretation of this result is that SIR4 masks the activation potential of SIR1 by binding to it. A similar interpretation followed the observation that certain LexA–RAP1 hybrids became activators in *sir3* or *sir4* mutants¹⁵.

Previous work has shown that when the *HMR-E* silencer is deleted for the ORC and RAP1 binding sites, and those sites were replaced by GAL4 binding sites, a GAL4 DNA binding domain (GBD)–SIR1 hybrid can establish silencing at *HMR*¹¹. This phenomenon, called targeted silencing, was taken to indicate that SIR1 is normally targeted to silencers by binding to ORC and/or RAP1 (ref. 11). Our results suggest that SIR1 is targeted by binding to ORC1. Therefore, we investigated whether a GBD–ORC1 hybrid (containing only the SIR1-interacting domain of ORC1, amino acids 5–267) was capable of targeted silencing. This ORC1 hybrid silenced a *TRP1* reporter gene almost as well as the GBD–SIR1 used originally¹¹. ORC1 targeted silencing, like SIR1 targeted silencing, was abolished in *sir2*, *sir3* and *sir4* mutants, and thus was dependent upon the presence of endogenous SIR2, SIR3 and SIR4 (data not shown). Targeted silencing by ORC1 was also abolished in a *sir1* mutant. This was in contrast to SIR1 targeted silencing, which did not depend on endogenous SIR1 (ref. 11; Fig. 3). These results demonstrate that SIR1 acts downstream of ORC1 in the silencing pathway.

To determine whether ORC1 targeted silencing depends on a

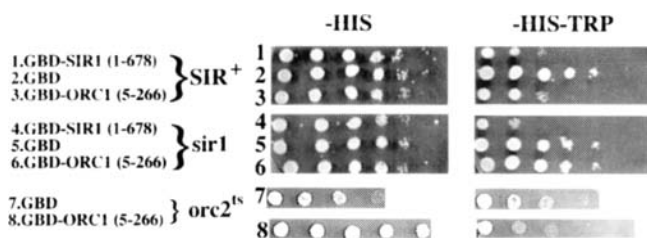


FIG. 3 Targeted silencing by GBD-ORC1 and GBD-SIR1. Rows 1–3 show results in YSB2, a *SIR*⁺ strain (ref. 11), and rows 4–6 show results in RS1172, a *sir1* derivative of YSB2. The plasmid present in each strain is indicated (GBD, GAL4 DNA binding domain). Cell cultures were serially diluted (1/10) 6 times, and 3 μ l of each dilution spotted from left (undiluted) to right (diluted to 1/10⁶) on media lacking histidine (-HIS) to measure the total number of cells plated, and on media lacking histidine and tryptophan (-HIS-TRP) to measure the amount of silencing. The lack of growth seen on -HIS-TRP medium in rows 1, 3 and 4 is due to targeted silencing. Rows 7 and 8 show results in YJS10-5B, an *orc2-1^{ts}* derivative of YSB2, grown at 23 °C. Here the spots (5 μ l) correspond to undiluted, 1/5, 1/10, 1/50 and 1/100 dilutions. The lack of growth on -HIS-TRP medium in row 8 signifies silencing by GBD-ORC1. Silencing by GBD-SIR1 could not be tested in the *orc2-1* mutant because SIR1 overexpression by this and other SIR1 plasmids was very toxic in the mutant. This is in marked contrast with the lack of any inhibitory effect of SIR1 overexpression in *ORC*⁻ strains.

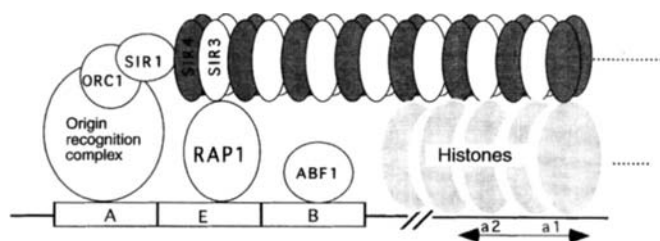


FIG. 4 A model for the establishment of silencing by SIR1 at the *HMR-E* silencer. The observed binding of SIR1 to ORC1 and SIR4 is indicated. Also shown is the binding of SIR3 to RAP1 (ref. 15), as well as the interactions of SIR3 and SIR4 with each other^{13,15} and with H3 and H4 (ref. 16). The model proposes that the seeding of the SIR3-SIR4 array begins at the silencer and spreads to silence nearby genes. For simplicity the drawing shows the spreading to be unidirectional; actually it is likely to be bidirectional¹⁸. The roles of ABF1 and SIR2 in silencing are not known and are therefore ignored in the model.

initiate the formation of silent chromatin. Whether SIR1 binds specifically to ORC at silencers or also to ORC bound at other chromosomal loci is not clear. The binding of SIR4 to SIR1 allows recruitment of that silencing protein. This brings SIR4 in close proximity to SIR3, which is bound by the neighbouring RAP1 (ref. 15; Fig. 4). As shown previously, SIR3 and SIR4 both homodimerize and heterodimerize with each other^{13,15}. The recruitment of both SIR3 and SIR4 to the silencer acts to seed an array of SIR3/SIR4 complexes that spreads from the silencer. The interactions of SIR3 and SIR4 with the N-terminus tails of histones H3 and H4 (ref. 16) are likely to cause the compaction of nucleosomes into a heterochromatic state. □

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1. Laurenson, P. & Rine, J. *Microbiol. Rev.* **56**, 543–560 (1992).
2. Bell, S. P. & Stillman, B. *Nature* **357**, 128–134 (1992).
3. Foss, M., McNally, F. J., Laurenson, P. & Rine, J. *Science* **262**, 1838–1844 (1993).
4. Bell, S. P., Kobayashi, R. & Stillman, B. *Science* **262**, 1844–1849 (1993).
5. Micklem, G., Rowley, A., Harwood, J., Nasmyth, K. & Diffley, J. *Nature* **366**, 87–89 (1993).
6. Fox, C. A., Loo, S., Dillin, A. & Rine, J. *Genes Dev.* **9**, 911–924 (1995).
7. Loo, S. et al. *Molec. Biol. Cell* **6**, 741–756 (1995).
8. Miller, A. M. & Nasmyth, K. A. *Nature* **312**, 247–251 (1984).
9. Pillus, L. & Rine, J. *Cell* **59**, 637–647 (1989).
10. Stone, E. M., Swanson, M. J., Romeo, A. M., Hicks, J. B. & Sternglanz, R. *Molec. cell. Biol.* **11**, 2253–2262 (1991).
11. Chien, C. T., Buck, S., Sternglanz, R. & Shore, D. *Cell* **75**, 531–541 (1993).
12. Hollenberg, S. M., Sternglanz, R., Cheng, P. F. & Weintraub, H. *Molec. cell. Biol.* **15**, 3813–3822 (1995).
13. Chien, C. T., Bartel, P. L., Sternglanz, R. & Fields, S. *Proc. natn. Acad. Sci. U.S.A.* **88**, 9578–9582 (1991).
14. Bell, S. P., Kobayashi, R. & Stillman, B. *Cell* **83**, 563–568 (1995).
15. Moretti, P., Freeman, K., Coodly, L. & Shore, D. *Genes Dev.* **8**, 2257–2269 (1994).
16. Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S. M. & Grunstein, M. *Cell* **80**, 583–592 (1995).
17. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. *Meth. Enzym.* **185**, 5–89 (1990).
18. Loo, S. & Rine, J. *Science* **264**, 1768–1771 (1994).

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ADDENDUM

Structural evidence for a two-step process in the depinning of the superconducting flux-line lattice

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Nature **376**, 753–755 (1995)

IN this Letter, we provided structural evidence for a two-step depinning process of the flux lattice in 2H-NbSe₂ but failed properly to reference the previous work of S. Bhattacharya, M. J. Higgins and A. C. Marley^{1–3}. In these papers, the occurrence of a regime intermediate between a pinned flux line lattice and a coherently moving one was inferred from transport and noise measurements on NbSe₂. We also regret that we inadvertently failed to acknowledge discussions with S. Bhattacharya. □

1. Bhattacharya, S. & Higgins, M. J. *Phys. Rev. Lett.* **70**, 2617–2620 (1993).
2. Bhattacharya, S. & Higgins, M. J. *Phys. Rev.* **B49**, 10005–10008 (1994).
3. Marley, A. C., Higgins, M. J. & Bhattacharya, S. *Phys. Rev. Lett.* **74**, 3029–3032 (1995).

functional ORC complex, we used an *orc2-1^{ts}* mutant. This mutant is temperature sensitive for growth and completely defective at silencing at the permissive temperature, as long as the *HMR-E* silencer is weakened by a deletion of its RAP1 binding site³. Surprisingly, ORC1 targeted silencing was totally unaffected by the *orc2-1* mutation (Fig. 3). Thus a small N-terminal domain of ORC1, when targeted to *HMR*, can establish silencing in the absence of a fully functional ORC. The silencing defects of *orc2* and *orc5* mutants indicate that ORC is important for HM silencing^{3,6,7}. However, the ORC2 independence of ORC1 targeted silencing suggests that the presence of an intact ORC is not an absolute requirement; rather, the localization of SIR1 to the silencers by means of ORC1 is the crucial ORC-dependent event.

Previous data have suggested that efficient establishment of the silent state at the *HM* loci requires both SIR1 (refs 9–11) and passage through S phase⁸. Our data suggest a simple explanation, that the SIR1-ORC interaction is regulated by an S-phase-dependent modification or stabilization of one of these proteins.

We present a model for the initiation of silencing at the *HM* loci (Fig. 4). During S phase, SIR1 is recruited to the silencer by its binding to ORC1. We propose that this binding acts as a trigger to

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Cell and tissue culture

Tools of the trade for those with the artistic touch and the scientific know how — this edition offers an economical bioreactor, a tissue compatibility ELISA, an enzymatic cultured-cell recovery system and microcarrier material

IMPERIAL LABORATORIES has announced the availability of cell lines for diagnostic and research purposes from their cell repository in the form of cryopreserved cell seed stocks. The company offers frozen cell stocks and recovered cells for customer orders. Cells can be provided as living cultures in exponential growth phase, monolayers in 75-cm² flasks in the case of anchorage dependent cells and at approximately 10⁶ cells ml⁻¹ in the case of those cells that grow naturally in suspension. The product range includes MRC-5, HeLa, HEP-2, McCoy and Vero, which are commonly used in diagnosis of pathogenic organisms and nearly a hundred diverse cell lines that have uses in a wide variety of biomedical research.

Reader Enquiry No. 100

The Axiovert 25 is a new inverted **tissue culture** microscope from Carl Zeiss. Its range of stages includes a new easy-to-clean, transparent-glass ceramic stage, which is designed to allow immediate identification of the objective underneath. It is said to have a compact, stable and ergonomic design, and can accommodate many types of culture container up to a height of 190 mm. This microscope should prove useful for observing, analysing and documenting the develop-

one objective, which obviates changing the objective while using the two contrast methods, allowing the selected magnification to be maintained. Axiovert 25 models are suitable for photography or video documentation in brightfield, phase-contrast and Varel, or for photographic or video documentation in brightfield.

Reader Enquiry No. 101

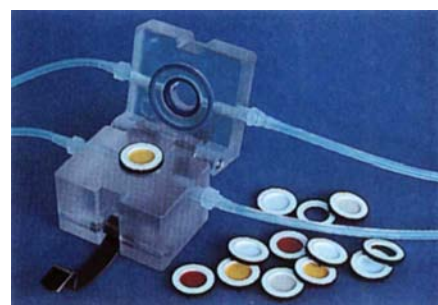
Bioreactors

The new E/Z Access Reactor with Omni-Vessel, an economical alternative to expensive **bioreactor** systems, has just been introduced by Bellco Glass. Designed for cell culture applications, including mammalian, plant and insect-cell culture, as well as bacterial fermentation, this system is versatile and compatible with Bellco overhead drives. The new functional system includes the vessel, a large flange headplate, a drive adapter, an overhead drive and an agitator. This is said to be a cost-effective system with a wide range of optional accessories to meet specific application requirements. The reactor is constructed of US FDA-approved materials and is said to conform to good manufacturing practice standards. The large-flange vessels are graduated to approximate volumes and

are available in three working volumes: 8-, 15- and 36-l. The 20-cm diameter, large-flange vessel is said to allow for easy set-up, operation, and disassembly, allowing for cleaning and autoclaving. The stainless steel headplate includes a quick-release clamp and a silicone O-ring. It is available in two configurations, with either 10 or 18 ports, and sanitary or NPT fittings also available. Optional accessories for the system include air-stone spargers, bubbler tubes, adjustable baffles, heating/cooling coils, heating blankets, probes, fittings, adapters and stainless steel tubes.

Reader Enquiry No. 102

Minucells and Minutissue allows the culture of epithelia under organotypical conditions. To mimic the functional bar-



In vitro epithelial growth carrier.

rier between the luminal and basal side of epithelial cells a gradient container for perfusion-culture experiments has been constructed. Epithelia grow on a 13- or 47-mm diameter carrier in which an individually selected cell support is placed for anchorage. Then the carrier is transferred with a forceps into the gradient culture container. The carrier separates the container into an upper and a lower compartment. An optimal differentiation of the epithelia is said to be obtained in long term culture experiments, when different media are perfused at the luminal and basal sides as observed in organ tissues.

Reader Enquiry No. 103

Using microcarriers it is now possible to grow adherent cells at high densities with the Miniperm **bioreactor** from Heraeus Instruments. This bioreactor is used for *in vitro* cultivation of animal, human and plant cells, and for the production of biomolecules released by the cells into culture medium. The cell cultivation system is designed to allow the growth of adherent cells in a small volume and at high density. For laboratory applications, this system produces optimum high yields. For example, when producing monoclonal antibodies or recombinant proteins, the amount required for research, development and diagnostic purposes can be produced. Among the anchorage-dependent cells are fibroblasts, keratinocytes and cells that are used for expression of genes



Zeiss' Axiovert 25 inverted tissue culture microscope.

ment of cell cultures. Two condenser systems are available for brightfield, phase-contrast and Varel methods. There are ICS optics, for brightfield, phase-contrast and incident-fluorescence, as well as a new spatial contrast method, from oblique brightfield to oblique darkfield. Phase-contrast and Varel are combined in



Microcarriers from Heraeus.

in the eukaryotic system (for example, BHK-21, CHO and COS cells). In order to cultivate these cells *in vitro*, they have to be immobilized on a carrier system. These carrier-cell complexes can be used in different biotechnological processes. Use of a microcarrier system leads to an increase of the culture surface and is said to result in higher cell numbers and better production results.

Reader Enquiry No. 104

Assay addendum

Autogen Bioclear now provides ELISA kits for assessment of **tissue compatibility and post-transplant monitoring**. sHLA-STAT B27 is said to be a rapid ELISA for typing HLA. Comparison with microlymphocytotoxicity yields a stated 99.2 per cent correlation for the ELISA. sHLA-STAT class I is a rapid sandwich ELISA for the quantitative measurement of soluble HLA class I antigens (sHLA) in serum, plasma and cell culture supernatant, and for use in the diagnosis of graft rejection (sensitivity is to 3 ng ml⁻¹). For detection of anti-idiotypic and isotypic antibodies to OKT3 there is the transSTAT-Ab, a membrane-based enzyme immunoassay kit. It is suitable for routine monitoring of antibody response in post transplant patients treated with OKT3.

Reader Enquiry No. 105

Endogen has acquired the biomedical products division of T Cell Diagnostics to bring together **mouse cytokine ELISA kits and human T-cell research reagents** within its organization. The company's range of specialty reagents has expanded to over 230 products with ELISA kits for human, mouse and pig cytokine quantitation. Also available are matched antibody pairs for human cytokine measurement, minikits for mouse cytokine quantitation, reagents and ELISA kits that are able to detect human T-cell surface markers and antibodies to human T-cell receptors. There are ELISA kits and reagents for the

detection of human, mouse and rat adhesion molecules, as well as a Bcl-2 ELISA kit for apoptosis research.

Reader Enquiry No. 106

Cell recovery

The Centritech Lab separation system is a new type of **centrifuge designed to handle animal cells and primarily cell cultures** in laboratory and pilot plants. This system is designed to concentrate cells and clarify media for purposes such as cell recycling, harvesting, washing, clarification and fractionation. This design makes it possible to rotate one end of a flexible tube while the other is standing still, thus avoiding the potential risk of culture infection. Separation takes place in a disposable separation bladder, installed in the narrow conical slit between the separation rotor wall and the support ring. The suspension is fed through the top inlet at one end of the bladder. Once inside the bladder, the cells, being heavier than the liquid, will under the influence of the centrifugal force move to the outside wall and slide to the bottom of the bladder, while the clarified liquid leaves the bladder via an exit at the top of the opposite end. At programmed intervals, the cell concentrate is automatically discharged through the concentrate outlet at the bottom of the bladder. The disposable separation insert consists of the bladder and the attached inlet and outlet tubes, all made from medical quality materials. The Centritech Lab system is designed for reproducible process set-up and easy scale-up. Both systems are flexible and can together handle flow rates from 0.05 l h⁻¹ up to 100 l h⁻¹.

Reader Enquiry No. 107

Becton Dickinson Labware has recently introduced a **non-enzymatic method to recover cells cultured** on the Matrigel Matrix, a reconstituted *in vitro* basement membrane. The matrix can be used to enhance differentiated functions of many cell types *in vitro*. With the aid of MatriSpense cell recovery solution, a matrix-free cell suspension can now be obtained by depolymerizing the matrix for one hour at 4 °C. A short incubation period at a low temperature is said to minimize intracellular biochemical changes during cell recovery, whereas the non-enzymatic polymerization process avoids digestion of the extracellular portions of cell-surface receptors and adhesion molecules. The MatriSpense solution is recommended for the recovery of cells from the matrix for subsequent biochemical analyses; particularly for the recovery of short-lived mRNAs or polypeptides and cell-surface molecules. It should also prove useful for replating cells onto a different matrix, if

sterile conditions are maintained, or for determining cell numbers, as long as cells do not clump.

Reader Enquiry No. 108

Cutting edge

The new Leica RM 2135 **rotary microtome** is designed for ergonomic comfort, a high level of safety and user friendly operation. This microtome should prove useful for paraffin and hard-sectioning techniques. Cross-roller bearings for horizontal and vertical specimen movement are used to ensure accurate, reproducible section thickness and optimum section quality, even when using high speeds and strong cutting forces. For the first time Leica is using this technique in one of its manually operated rotary microtomes. A



Leica RM 2135 rotary microtome.

spring-lever, mass-balanced system offsets gravitational forces during the sectioning process. This is said to allow a smooth and easy turn of the handwheel to obtain a uniform stroke for excellent chatter-free sections. As a result, the smooth running handwheel creates stress-free working conditions and repetitive motion disorders are reduced to a minimum. The handwheel is lockable in all positions by simply relocating a lever, making the instrument safe to use when manipulating the knife or the specimen. Another safety feature is the 70-mm vertical stroke length, which promotes safety during specimen changes and other manipulations in front of the knife edge. Furthermore, it allows the sectioning of large samples due to a maximum sectioning range of 69 mm. Specimen retraction, a new feature, is designed to protect the knife and the sample from damage. The coarse feed wheel is located ergonomi-

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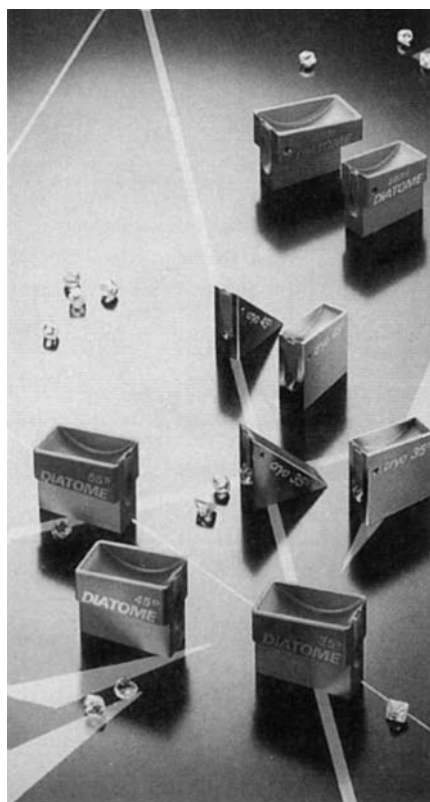
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READER ENQUIRY NO. 10

wheel is located ergonomically to ensure fatigue-free work. The unit can be ordered according to the user's preference, with the coarse feed wheel rotating clockwise or counterclockwise.

Reader Enquiry No. 109

Diatome offers its diamond knife to meet both **biological and industrial cutting requirements**, regardless of the type of material being cut. The company offers six



Diamond knives for biological cutting.

knife types for cutting at ambient or low temperatures, manufactured with three different inclination angles to meet a variety of needs. All of knives are highly hydrophilic and are designed for long life.

Reader Enquiry No. 110

Cell analysis

Immunetics has developed a **cell-adhesion flow chamber** for use in studies of cell-cell interactions. Using this chamber, interactions between cell types can be observed, captured and analysed by videomicroscopy. The chamber is designed to provide true laminar flow, permitting the user to pump a cell suspension between two parallel surfaces at a controlled rate. The lower surface is formed from a precision-milled and optically polished acrylic plate, whereas the upper surface is provided by a microscope culture slide on which a monolayer of cells has been grown. Once pumped into the chamber, cells in suspensions flow

past cells adhering to the slide in an even, lamellar flow pattern. The slide is held tightly to the device by integral vacuum ports. For compatibility with conventional microscope equipment the unit has been designed to be manipulated on an inverted stage equipped with a 96-well microplate holder. The flow chamber can be used to research cell adhesion to vascular and endothelial tissues, studies of metastatic cells and the cellular immune system.

Reader Enquiry No. 111

Oxford Glycosystems offers the services of its analytical services group to provide a broad range of **carbohydrate analyses to determine carbohydrate composition, glycan profiles and oligosaccharide structure**. Glycosylation analysis is important in monitoring antibody production, quality control of recombinant products, optimization of cell culture conditions and structure/function studies. Confidential analyses are independently validated, strictly controlled and can be customized to meet the requirements of specific applications. Results are obtained quickly and accurately by carbohydrate chemist using reliable instrumentation and well developed methodology. Data can be provided in a variety of formats and help with data interpretation is also available.

Reader Enquiry No. 112

The Endohm-Snap chamber from WPI has been designed specifically for use with Costar Snapwell culture cups to **measure tissue resistance**. The culture cups may be transferred from their culture wells to the Endohm chambers for more accurate measurements than might be possible with hand-held electrodes. The new Endohm-Snap is equipped with a planar electrode for low-resistance tissue measurement and is configured to allow a more uniform current density to flow across the membrane. Concentric electrodes are situated above and below the membrane, reducing background resistance from 150 Ω to less than 5 Ω . According to the manufacturer, the fixed-electrode geometry reduces the variation of readings on the same sample from 10–30 Ω , using the Evom hand-held electrode, to 1–2 Ω , using the Endohm-Snap electrode. The chambers, together with the Evom epithelial volt ohmmeter, should prove to be an accurate and economical method for endothelial resistance measurement. Although the Endohm-Snap was designed for use with the Costar Snapwell cup, the



Endohm-Snap chambers from WPI.

chamber will accept almost all makes of 12-mm to 24-mm culture cups.

Reader Enquiry No. 113

Enhancing culture

PAA Laboratories has introduced Viralex trypsin, which has been treated by a ultra-violet process to **inactivate parvovirus, yet retain the cell-dissociation activity of the trypsin**. Mycoplasma and parvovirus are common contaminants of cell-culture-grade trypsin manufactured from porcine or bovine pancreases. Traditional methods of inactivation of these contaminants, such as γ -irradiation, are not effective due to the high resistance of parvovirus and the deleterious effect of the treatment on the enzymatic cell-dissociation activity. This product is parvovirus inactivated, but still retains the cell dissociation activity of the trypsin.

Reader Enquiry No. 114

Bibby Sterilin has developed the Fibra-Cel **microcarrier material**, which is said to offers enhanced performance for a wide range of anchorage-dependent cell culture applications. The carriers consist of 6-mm discs of non-woven polyester, each bound to a polypropylene screen, which acts as a weighting device. The surfaces are tissue culture treated providing conditions for maximum cell attachment and growth. The discs are easy to use and simple to scale up. They form a dramatically enlarged surface area that is ideal for the demands of high-volume production of biological macromolecules from cell cultures. Fibra-Cel may provide cell growth to densities in excess of 5×10^7 cell ml^{-1} , considerably greater than that achieved by conventional roller bottle or even microcarrier techniques. This can be achieved with low cell-seeding densities, down to a stated one per cent of the final density. In addition to increasing surface area, Fibra-Cel is designed to boost productivity by protecting fragile cells from

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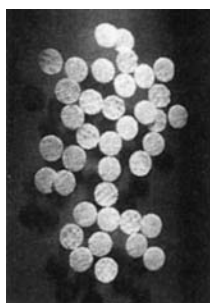
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shearing damage, allowing them to grow within the fibre disc. Not only are culture conditions optimized, but the system is flexible and can be utilized in existing applications with ease. For packed-cell continuous cultures, the discs can be immobilized in a compartment and perfused with nutrient medium. Product secreted into the medium can then be harvested, cell-free for downstream processing and purification. In a stirred system, the discs can be easily mixed and will settle to allow harvesting or removal of the supernatant; in a roller systems, the surface area for cell growth can be increased by incorporating the discs within the roller bottle. This product offers high cellular attachment and growth in serum-free media. Fibra-Cel should prove useful for the growth of cell lines where secreted or excreted proteins are required, and has applications in vaccine and hybridoma production, cellular protein culture and cell biology research.



Fibra-cel discs.

Reader Enquiry No. 115

Miscellaneous

Metachem Diagnostics has announced the availability of the StemSep lineage depletion system for the **enrichment of progenitor cells**. StemSep is a magnetic system for negative selection of human haematopoietic progenitors. In this system, the unwanted cells are labelled with a cocktail of antibodies that bind to the separation surface, thus the harvested target cells have not been labelled with antibodies or magnetic particles. Concerns over whether to remove antibodies or particles are not an issue and there are no limitations on further antibody labelling for cell analysis, activation and the like (antibody binding sites have not been blocked). The cells are ready for analysis using flow cytometry and haematopoietic colony assays. The system can be used with poor samples as the stickiness of unwanted dead cells, monocytes and granulocytes does not cause a problem.

Reader Enquiry No. 116

Shandon has introduced a new edition of Academy **cytology and histology training software**. The software is a Windows programme that is divided into 16 interactive modules, which discuss in detail the tissues and organ systems of the body. More than 170-colour photomicrographs and 20 drawings illustrate this comprehensive presentation. Users can progress at their

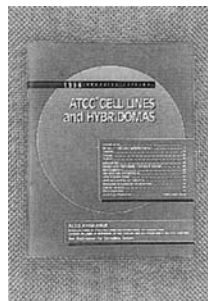
own pace and test their knowledge in review test sections.

Reader Enquiry No. 117

Polysciences has recently introduced SectionLock **silanated slides for *in situ* hybridization** procedures. These slides are treated with a specially formulated aminoalkylsilane to permit covalent coupling of tissue samples to the slide surface. Embedded tissue sections will stay on the slide even during long digestion and hybridization procedures. The slides come in ready-to-use, pre-cleaned, colour-coded boxes of 72.

Reader Enquiry No. 118

American Type Culture Collection (ATCC) has announced the availability of the 1996 ATCC catalogue for ordering **cell lines and hybridomas**. This catalogue contains listings of over 3,000 different cell lines and hybridomas available from ATCC. The general alphabetical index is supplemented by special indexes to help locate desired lines by species, tissues, tumors and monoclonal antibody (Mab) specificity. In addition, there is information describing several new cell-culture related products available from ATCC, including genomic DNA from human cell lines, Culture-Check (a microbial detection kit for contamination of cell lines), pre-adapted serum-free cell lines and a mycoplasma detection kit.



ATCC Catalogue.

Reader Enquiry No. 119

To better understand the **interface between nerve fibres with plastic, silicon or metal**, Fraunhofer has set up a new type of culture laboratory where biologists and solid-state physicists will work side-by-side. They will test the biocompatibility of microelements, such as electrodes and sensors, and develop processes by which cells will adhere to silicon. In addition, the scientists are cultivating cell strains receptive to implants. Further developments, such as flexible plastic structures that will gently wrap themselves around nerve fibres and micro-structures on which cells will attach and grow, are awaiting testing. Ultimately, it is hoped that hard chips, which must remain dry, will be linked with soft cells requiring an aqueous environment. Hardware integrated with wetware may ultimately provide improve artificial implants. Important biotechnical combinations are expected in the future, such as restoring sight to the blind by means of a

retinal prosthesis or, for example, stimulating the muscles of paraplegics to carry out deliberate movements.

Reader Enquiry No. 120

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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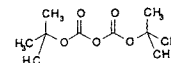
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